VOLUME NINETY SIX

ADVANCES IN PARASITOLOGY

Echinococcus and Echinococcosis, Part B

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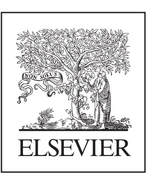

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PREFACE

Our aim in this thematic volume of *Advances in Parasitology* is to provide a complete synthesis of what is known about the cestode parasite *Echinococcus* and the diseases it causes, echinococcosis (hydatid disease). It builds on the success of two previous volumes 'The Biology of *Echinococcus* and Hydatid disease' and '*Echinococcus* and Hydatid Disease' published by Allen and Unwin and CAB International, respectively, and details the major advances that have taken place since. The 10 chapters demonstrate that in addition to its medical, veterinary and economic significance, *Echinococcus* is an intriguing biological phenomenon. They detail the major advances that have taken place during the last 20 years, particularly in our understanding of taxonomy, genetic variation, developmental biology, host–parasite relationships, geographic distribution and host range, diagnosis, control and clinical management. In addition, we have included a chapter covering historical aspects of echinococcosis that highlights major contributions to knowledge about the parasite and the diseases it causes. In this respect, four doyens of the field and contributors to the previous volume have passed away in recent years: Desmond Smyth, Michael Gemmell, Robert Rausch and Rudolf Ammann. We are therefore pleased to have the opportunity that this volume provides to detail and highlight the major contributions they have made to the field.

Although major advances have been made in research on *Echinococcus* and echinococcosis, many questions remain, particularly in the areas of developmental biology and host–parasite relationships. Control efforts have had limited impact globally, and *Echinococcus* is an emerging problem in some parts of the world. As with many zoonoses, control is hampered by anthropogenic factors that influence both domestic and wild cycles of transmission.

The overriding theme of the book is that a comprehensive understanding of the biology of *Echinococcus* is essential for the effective treatment and control of echinococcosis. The links between laboratory knowledge and field applications are emphasized throughout the book. Consequently, we hope that research workers, teachers and students of parasitology, clinicians

and field workers will find this work an indispensable source of information, but that it will also provide a model for the integration of basic and applied research in parasitology.

Andrew Thompson, Peter Deplazes and Alan Lymbery

CHAPTER ONE

Immunology of Alveolar and Cystic Echinococcosis (AE and CE)

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Abstract

Cystic and alveolar echinococcosis are severe chronic helminthic diseases caused by the cystic growth or the intrahepatic tumour-like growth of the metacestode of *Echinococcus granulosus* or *Echinococcus multilocularis*, respectively. Both parasites have evolved sophisticated strategies to escape host immune responses, mainly by manipulating and directing this immune response towards anergy and/or tolerance. Recent research studies have revealed a number of respective immunoregulatory mechanisms related to macrophages and dendritic cell as well as T cell activities (regulatory T cells, Tregs). A better understanding of this complex parasite—host relationship, and the elucidation of specific crucial events that lead to disease, represents targets towards the development of novel treatment strategies and options.

1. BRIEF INTRODUCTION TO ALVEOLAR ECHINOCOCCOSIS AND CYSTIC ECHINOCOCCOSIS

1.1 The parasites, their basic biology

The small tapeworm genus *Echinococcus* encompasses multiple species and genotypes, two of them representing considerable health risks to humans: *Echinococcus multilocularis* causing 'alveolar echinococcosis' (AE), and *Echinococcus granulosus* sensu stricto causing 'cystic echinococcosis' (CE). CE in humans can be, although less frequently, also inflicted by a few closely related species including e.g., *Echinococcus ortleppi*, *Echinococcus canadensis*, and *Echinococcus vogeli*, among others. All classified *Echinococcus* species and genotypes have a two-host life cycle, including a sexual (adult) stage in the intestine of carnivorous definitive hosts and a larval stage in the tissues of mostly noncarnivorous or omnivorous intermediate hosts (Eckert and Deplazes, 2004). The larvae, also called metacestodes, comprise various forms in dependence of the species, but they are all structurally based on the same principle of a fluid-filled vesicular cyst, incorporating an inner germinal layer

(GL), and, in case of maturation, multiple protoscoleces, and an outer laminated layer (LL). Transmission of infection from definitive to intermediate hosts occurs primarily via parasite eggs fecally shed by the definitive host. Such eggs contaminate food and water and can thus perorally enter the gastrointestinal tract of intermediate hosts. Predating infected intermediate hosts by carnivorous definitive hosts allows the protoscoleces present inside the parasitic vesicles to get into the gastrointestinal tract of definitive hosts and to maturate there into adult stage tapeworms, which closes the parasite's life cycle.

1.2 Biology of infection in intermediate hosts

Echinococcus multilocularis: To develop AE, appropriate intermediate hosts, after getting perorally infected with parasite eggs, allow the parasitic early stage larva, the oncosphere to migrate to the hepatic tissue, where transformation into a GL with the shape of a small vesicle occurs within the first few days postinfection; within approximately another 7–10 days, this vesicle has synthesized an outer LL (Gottstein et al., 1992) that appears crucial to protect the parasite from host's early innate-specific or subsequent specific immune reactions. From this point on the different *Echinococcus* species vary considerably regarding the anatomy and thus also pathogenesis of the developing and maturating metacestode. For *E. multilocularis*, the primary vesicles remain small in size and rapidly start to form small external buds that proliferate as either still connected or sometimes detached secondary vesicles. This small vesicular reproduction and multiplication is continuous, and its velocity is dependent upon the nature of the periparasitic host reaction (see below). The GL of each vesicle may internally produce brood capsules, which differentiate into protoscoleces, which will be responsible for the development of the adult worm in the intestine of the definitive hosts after their ingestion of the intermediate host as described earlier.

Microscopically, viable and proliferating AE lesions are characterized by an extensive conglomerate of small vesicles, each consisting of an inner GL composed of a thin coat of syncytial cells and multiple other cell types, but particularly, beside muscle cells, glycogen storage cells and others, a relatively large amount of primary 'stem' cells; the GL is surrounded by a thin acellular PAS-positive LL (Gottstein et al., 1992). Parasite proliferation is usually accompanied by a periparasitic granulomatous host response, including vigorous synthesis of fibrous and connective tissue in the close vicinity of the LL of the metacestode. The metacestode tissue expresses its highest growth activity in the peripheral region of the parasite tissue, while

central parts frequently demonstrate necrotic degenerations. In infected humans, the *E. multilocularis* metacestode-induced lesion appears macroscopically as a dispersed mass of fibrous tissue with a conglomerate of scattered vesiculated cavities with diameters ranging from a few millimetres to a few centimetres in size (hence the word 'alveolar') (Eckert and Deplazes, 2004). In advanced chronic cases, a central necrotic cavity may form, which may reach up to 2 decimetres in size and contains a viscous fluid; bacterial superinfection may occur within the cavity, usually when there is communication with the bile ducts (Stojkovic et al., 2014). The lesion often contains scattered or isolated zones of calcification, typically within the metacestode tissue and not in the periphery as typically found in CE (Stojkovic et al., 2014).

Echinococcus granulosus: For CE, the initial phase after peroral infection with *E. granulosus* eggs starts similar to that of *E. multilocularis*. The oncospheral migration of the parasite, however, ends in the liver in only approximately 60% of the cases (Brunetti et al., 2010). In more than 20% the final target organ is the lung, and the remaining 20% of oncospheres end up at various other sites, including the brain, bones, muscles, kidney, etc. (Brunetti et al., 2010). Wherever the oncosphere homes, the differentiation process into the metacestode stage will also start by its transformation into a small vesicle. The *E. granulosus* LL is probably formed by a single type of mucin backbone (Diaz et al., 2015). The LL carbohydrates from both species have been found to interact selectively with the Kupffer cell receptor (KCR) expressed in rodent liver macrophages (MØs), highlighting the ancestral adaptations to rodents as intermediate hosts and to the liver as infection site (Hsu et al., 2013).

The *E. granulosus* vesicle does not have the potential of external budding as *E. multilocularis*. In contrast, it has a tremendous potential to increase concentrically in size and to form the classical fluid-filled 'hydatid cyst' (Brunetti et al., 2010). The inner GL looks very similar to that of *E. multilocularis*. This GL has the potential to develop — internally within the cyst — two structures: (1) it can produce the 'brood-capsules', which themselves will internally produce protoscoleces. In CE, these protoscoleces, in addition to their capacity to generate the adult worm in definitive hosts, have also the capacity to rapidly transform into a small vesicle. Such a protoscolex-derived vesicle can subsequently develop and maturate into a secondary cyst in the intermediate host. The GL (2) can produce 'daughter vesicles', a feature that occurs especially after aggression of the primary cyst wall; such daughter vesicles (some authors call them also cysts)

may also, but very rarely, be produced externally to the primary cyst (da Silva, 2011; Rogan et al., 2015). A very thick LL represents the outer part of the cyst. Daughter vesicles also synthesize a surrounding LL, which, in case of primary cyst rupture, will protect them against host immune aggression.

A viable and thus metabolically active hydatid cyst evokes a cellular and humoral host response that leads to the formation of a host-derived adventitial fibrous capsule that contributes to the host's control of cysts growth. A totally efficient periparasitic immunity and/or chemotherapy and/or physical aggression (puncture, chemical agents) can lead to cyst degeneration, which leads to an increasing calcification of the periphery of the cyst, one of the typical features found in imaging procedures (Rogan et al., 2015).

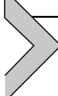
1.3 Alveolar echinococcosis and cystic echinococcosis represent very different diseases

Although *E. multilocularis* and *E. granulosus*, from the genetic point of view, are very closely related species of the same genus (Thompson and Jenkins, 2014), from the clinical viewpoint, AE and CE are entirely different regarding symptoms, course of disease and prognosis, to the extent that clinicians should consider the two parasitic infections as distinctly different diseases (Kern, 2010; Brunetti et al., 2010; Stojkovic et al., 2014).

AE: Patients with symptomatic AE present liver lesions ranging in size between a few millimetres up to decimetres. A classification of the different types and stages of AE (Kern et al., 2006) facilitates a standardized registration of AE and thus to carry out retro- and prospective clinical and epidemiological studies. Most frequent symptoms at diagnosis comprise hepatomegaly and cholestatic jaundice, and symptoms of liver abscess, portal hypertension and Budd–Chiari syndrome. However, the infection may be asymptomatic for long time [approximately 5–15 years (Eckert and Deplazes, 2004)] and in countries where medical imaging techniques are easily available or where ultrasound mass screening is organized, hepatic lesions are incidental findings (Bartholomot et al., 2002; Chauchet et al., 2014). The disease starts frequently with nonspecific clinical signs such as epigastric pain or biological signs of anicteric cholestasis that can be incidentally found. The infiltrative and compressing tumour-like growth of the metacestode tissue is the main cause of disease. This includes complications such as bile duct destruction due to invasion of large biliary

ducts at the liver hilum with or without cholangitis. Further damage of liver parenchyma can cause secondary biliary cirrhosis, portal hypertension and hepatic dysfunction. Obstruction of vascular walls causes vascular occlusion/thrombosis of the portal veins, leading to portal hypertension of the hepatic veins, and/or to the Budd–Chiari syndrome, and/or of the vena cava and/or the right atrium. Invasion of vessels can lead to haematogenic spread of infection most likely via microvesicles released by the metacestode (Stojkovic et al., 2014). Finally, in advanced cases, the superinfection of central necrotic cavities within the lesions may mimic a bacterial liver abscess. Invasion of neighboring organs and tissues (most frequently of the diaphragm) occurs in nearly 1/5 patients (Piarroux et al., 2011). Secondary distant metastasis formation can occur late in brain, spine, lung, bone and rarelyother sites (Kern et al., 2003; Piarroux et al., 2011). The growth rate of the metacestode tissue is usually slow in immunocompetent patients. Analysis by CT scans indicated an average volume increase of 15 ml/year for progressive forms of AE (Aydinli et al., 2008; Kantarci et al., 2012; Stojkovic et al., 2014). Extrahepatic AE without hepatic involvement is rare but possible (4% in a series of 362 French patients) (Piarroux et al., 2011).

CE: In patients with CE, the cysts grow but do not spread or metastasize and signs and symptoms are directly related to the space-occupying situation. Increasing pressure on essential organic structures such as bile or blood vessels variably induces hepatomegaly, chronic cholestatic jaundice (and subsequently biliary cirrhosis), portal hypertension and Budd–Chiari syndrome. Biliary complications and secondary bacterial superinfection and abscess formation because of communication with bile ducts are the most frequent complications (Brunetti et al., 2009). Patients with CE-affected lungs present with chronic cough, haemoptysis, bilioptysis, pneumothorax, pleuritis, lung abscess and parasitic lung embolism (Stojkovic et al., 2014). Cyst location in heart or brain can cause complete heart block and sudden death, or various neurologic symptoms, respectively. A cyst may rupture and spill its content into surrounding sites; fissure and rupture may also cause allergic reactions, with various symptoms from urticaria, angioedema to fatal anaphylactic shock. Spilled protoscoleces can develop into secondary cysts, which is the most frequent reason for relapses. Most cysts long remain asymptomatic and are disclosed incidentally or during systematic ultrasound mass screening (Frider et al., 2001; Yang et al., 2006).



2. BACKGROUND IMMUNOLOGY-BASED ON MURINE STUDIES

2.1 Innate immunity, macrophages, dendritic cells and susceptibility to infection

AE: Innate immunity is the first line of defence of a host to tackle infectious organisms, and this first line primarily depends on the immunogenetic background of a permissive host, but additionally also on other genetic and physiological parameters found in different intermediate host species and strains. In this respect, a recent study demonstrated that immunocompetent Wistar rats perorally infected with *E. multilocularis* eggs were resistant to infection, whereas intraperitoneal inoculation of nonactivated oncospheres resulted in a successful establishment of a metacestode and thus disease (AE) (Armua-Fernandez et al., 2016). T cell-deficient athymic nude rats were resistant to peroral parasite egg infection as well, whereas dexamethasone-(DMS)-immunosuppressed animals became susceptible upon the same mode of infection. One of the major conclusion of the study, based on the finding of higher amounts of MØs and NK cells in nude rats, was that innate immune mechanisms appear to be responsible for the provision of resistance to peroral infection in rats, and that simultaneously factors affected by DMS appear to be involved in regulation of resistance versus susceptibility (Armua-Fernandez et al., 2016). Matsumoto et al. (2010) studied susceptibility of various rodent host strains with regard to metacestode proliferation and fertilization. The authors found that the rate of parasite establishment was highest in DBA/2, followed by AKR/N, C57BL/10 and C57BL/6 mice, whereas gerbils harboured few parasite foci. The course of larval development was most advanced in DBA/2 mice with mature protoscolex formation at 16 weeks p.i., followed by AKR/N harbouring metacestodes with sparsely distributed immature protoscoleces (Matsumoto et al., 2010). On the other hand, C57BL/6 and C57BL/10 mice had infertile metacestodes without any protoscolex formation. As most human AE cases do not develop protoscoleces during infection, studies based on, e.g., C57BL/6 mice may most likely best reflect human AE (Matsumoto et al., 2010). In mice, multiple genetic factors (quantitative trait loci (QTLs)) were found to regulate host susceptibility or resistance to *E. multilocularis* infection, and QTLs which are associated with establishment of the parasite in the liver were distinct from those for protoscolex development, indicating that different host factors are engaged at each developmental stage of the larval parasite (Nakao et al., 2011). These studies followed actually earlier

ones that had already indicated that differences in murine immune responses occur in different mouse strain exhibiting various degrees of susceptibility versus resistance (Liance et al., 1984; Gottstein et al., 1994; Guerret et al., 1998). Similarly, it was shown that an overall impairment of cell-mediated immunity is followed by an increased susceptibility for *E. multilocularis* metacestode proliferation (Baron and Tanner, 1976) yielding a fully uncontrolled parasite growth in severely immune-deficient animals such as in SCID mice (Playford et al., 1992)) and in nude mice (Dai et al., 2004). An increase of susceptibility, associated with a decrease of delayed-type hypersensitivity, was also observed in mice infected with *E. multilocularis* and treated with an immunosuppressive drug, cyclosporine, which interferes with the interleukin-2 (IL-2) production in T cells (Liance et al., 1992).

During infection, there is a strong interaction potential between phagocytic host cells and the parasite surface. However, such host cells are hampered in their function by downregulation of several processes affecting their maturation and antigen presentation properties. It has been shown that the LL protects the GL from nitric oxide (NO) produced by periparasitic MØs, and that it can also prevent immune recognition by surrounding T cells (Vuitton and Gottstein, 2010). Furthermore, iNOS-deficient mice exhibit a significantly lower susceptibility towards experimental infection, which strongly suggests that the high-periparasitic NO production by peritoneal exudate cells contributes to periparasitic immunosuppression (Dai and Gottstein, 1999; Dai et al., 2003; Andrade et al., 2004). Tackling a deeper insight into MØ functional impairment, it was shown that MØ from AE-infected mice (AE−MØ) exhibited a reduced ability to present a conventional antigen (chicken ovalbumin) to specific responder lymph node T cells when compared to normal MØ (Mejri and Gottstein, 2006). As MØ from mice with AE fully maintained their capacity to appropriately process antigens, a failure in T cell receptor occupancy by antigen−MHC (major histocompatibility complex) complex or/and altered costimulatory signals could be excluded. The CD80 and CD86 costimulatory molecules involved in T cell stimulation by MØ appeared unchanged, whereas CD40 was downregulated and the adhesion molecule CD54 was slightly upregulated (Mejri and Gottstein, 2006). Overall, the antigen-presenting activity of AE−MØ appeared to trigger an unresponsiveness of T cells leading to the suppression of their clonal expansion during the chronic phase of *E. multilocularis* infection in mice (Mejri and Gottstein, 2006). Conversely, treatment of *E. multilocularis*-infected mice with recombinant alpha-interferon 2α (IFN-α-2α) partially abrogated the immunosuppressive traits

and pathway of infection (i.e., decreased the IL-10 production and restored phagocytosis and oxidative metabolism of MØs) (Godot et al., 2003). Experimental evidence showed that the type of the primary immune response towards infection, initially Th1-based, became progressively Th2-oriented (Mejri et al., 2011b) during the progressive growth of the metacestode. Concomitantly, intraperitoneal dendritic cells (DCs) and T cells isolated at the late stage of infection expressed relatively high levels of transforming growth factor-beta (TGF-β) mRNA, while in peritoneal DCs IFN-γ mRNA, and the surface expression of the major costimulatory molecules CD80, CD86, CD40 and the MHC class II (Ia) molecules were downregulated (Mejri et al., 2011a). Overall, evidence that the intraperitoneally proliferating metacestode impedes the maturation and activation of DCs was accumulated. Therefore, DCs in *E. multilocularis*-infected mice can be classified as tolerogenic cells, and moreover, as cells with suppressive features based upon their high level of TGF-β expression.

CE: Based upon in vitro and in vivo (mice) studies with *E. granulosus*, it was shown that the LL of the hydatid cysts was involved in downregulating nitric oxide production (Steers et al., 2001) and thus contributed to an impairment of proinflammatory processes. *Echinococcus granulosus* oncospheres and subsequently cysts escape host's immunosurveillance by interfering with monocyte differentiation and by modulating DC maturation (Riganò et al., 2007). Few studies investigated the host's innate immune response following different stages of *E. granulosus* infection. Pan et al. (2013) investigated innate and adaptive immunity at 30, 180, 360 days postinfection (dpi) in mice infected with *E. granulosus*. The authors described at 30 dpi an increase in the number of $CD11b^+$ (predominantly MØs) and $CD11c^+$ (predominantly tissue DCs and MØs) antigen-presenting cells (APCs), which was also accompanied by a slight downregulated expression of the antigen-presenting MHC class II molecule (Pan et al., 2013). The response of DCs to mucin-based gel of the LL of *E. granulosus* was studied in vitro and in vivo (Casaravilla et al., 2014). In vitro, LL particles induced an unusual activation state characterized by upregulation of CD86 without concomitant upregulation of CD40 or secretion of cytokines (IL-12, IL-10, tumour necrosis factor-alpha (TNF-α), and IL-6). When added to Toll-like receptor (TLR) agonists, LL particles potentiated the upregulation of CD86 and IL-10 secretion while inhibiting CD40 upregulation and IL-12 secretion. In vivo, LL also caused upregulation of CD86 and inhibited the CD40 upregulation in DCs, thus indicating that DCs responded to the LL mucin meshwork with a 'semimature' activation phenotype (Casaravilla et al., 2014).

2.2 The role of different Th cell types (focusing on Tregs and Th17) and respective cytokines/chemokines

AE: The periparasitic granuloma in the liver is a major characteristic of AE pathology, and granuloma formation is mainly orchestrated by the pattern of cytokines interacting with immune and nonimmune cell interaction in the periparasitic tissue. By using an intraperitoneal infection mouse model, it has been shown that the type of the primary immune response towards infection appears initially Th1-oriented, but subsequence becomes progressively Th2-oriented (Mejri et al., 2011a), thus leading to the conventional chronic stage of AE. However, in a recent study with an intrahepatic infection mouse model (Wang et al., 2014a,b), IL-4 expression could be evidenced very soon after primary infection of mice (within the first 2—8 days postinfection). Conclusively, the major Th2-orienting cytokine appeared to be present earlier than anticipated in previous studies. Overall, accumulating documentation of differences in specific characteristics of early and late stage immune responses between primarily and secondarily infected mice may indicate that the two infection modes may be more significant than previously anticipated.

Experimental infection (intraperitoneal) of mice resulted in a $CD4^+$ T cell hyporesponsiveness associated with differentiation of Treg cells (Mejri et al., 2011b). The most widely described suppressor T cells are $CD4^+CD25^+$ $FoxP3^+$ T cells that express high levels of cell surface-associated TGF-β. The high expression level of TGF-β in *E. multilocularis*-infected mice seemed to largely contribute to the development of regulatory $CD4^+CD25^+$ $Foxp3^+$ T cells and $CD8^+CD25^+$ $Foxp3^+$ T cells (Mejri et al., 2011a).

Tregs appear thus as key immunomodulators in murine AE, associated with impaired MØ and DC functions. In the frame of very recent investigations on immunomodulation in AE, the role of FGL2 (fibrinogen-like protein 2) as another key parameter in the Treg-dependent downregulation of periparasitic immunity was addressed. FGL2 is a member of the fibrinogen-related superfamily, is highly expressed in Tregs and has an important role in Treg cell effector function (Levy et al., 2000). Microarray studies showed that FGL2 mRNA level was significantly upregulated in the liver of *E. multilocularis* perorally infected mice (Gottstein et al., 2010). In *E. multilocularis*-infected FGL2-deficient mice (as compared to infected wild-type mice), a significantly lower parasite load and a reduced parasite proliferation activity was observed, associated with increased T cell proliferation in response to ConA, reduced Treg numbers and function, relative Th1

polarization and increased B cell numbers and DC maturation (Wang et al., 2015a). It became also evident, for the first time, that FGL2 was involved in immune regulatory processes favouring larval helminth parasite survival, and that IL-17A contributed to FGL2 regulation. By promoting Treg cell activity, FGL2 appears thus as one of the key players in orchestrating the immunomodulation that permits chronic AE (Wang et al., 2015b).

As mentioned earlier, the main cytokines for Tregs involved in immune tolerance are IL-10 and TGF-β, and these have been largely studied in the past three decades. The metacestode actively achieves a tolerance status through the induction of regulatory cytokines IL-10 and TGF-β (Mejri et al., 2009). A more detailed subsequent study in mice suggested that, in addition, TGF-β might play an important role in liver fibrosis through its downstream Smad signaling pathway (Wang et al., 2013).

More recently, La et al. (2015) addressed the relationship between programmed cell death-1/programmed death ligand-1 (PD-1/PD-L1) pathway and Tregs at different stages of AE. PD-1 is a coinhibitory receptor on T cells that plays a major role in exhaustion, a dysfunctional state of effector cells caused by antigen persistence; exhausted T cells present defects in effector function including impaired proliferation, and reduced cytotoxic capacity and cytokine production including IL-10 and TGF-β. These defects can be partially restored by blocking the interaction between PD-1 and its ligand PD-L1. During the middle to late stage of infection (day 30 to day 330) of AE in the murine model, the percentages of PD-1^+ $CD4^+$ $CD25^+$ Tregs and PD-$L1^+$ $CD11c^+$ MHC class II^+ DCs together with levels of PD-1, PF-L1, Foxp3, IL-10 and TGF-β mRNA increased significantly and were maintained at high level. The reduced proliferation of T cells isolated from *E. multilocularis*-infected mice and their increased production of IL-10 and TGF-β was reversed when anti-PD-L1 antibody was added. High expression of PD-1/PD-L1 may thus play an important role in promoting $CD4^+CD25^+$ T cell expansion, such maintaining peripheral tolerance and immune evasion during chronic AE through the means of T cell exhaustion.

The most recent studies on Tregs in murine AE have focused on $CD4^+CD25^+$ Tregs and, as shown earlier, have confirmed them as major players in the tolerance state observed in AE. However, the immunosuppressive role of $CD8^+$ T cells was evoked as soon as the first immunological studies on *E. multilocularis* infection had been conducted, mainly because of their abundance in the periparasitic infiltrate both in humans with severe infections (Vuitton et al., 1989) and in susceptible mice (Bresson-Hadni et al., 1990). Generation of $CD8^+$ 'suppressor T cells' under the influence

of *E. multilocularis* protoscoleces could be demonstrated in in vitro experiments (Kizaki et al., 1993a, 1993b). More recently, high expression levels of Foxp3 mRNA by $CD8^+$ as well as $CD4^+$ peritoneal T cells were described in the intraperitoneal model of *E. multilocularis* infection (Mejri et al., 2011a). While $CD4^+CD25^+$ $Foxp3^+$ T cells are well known for their role in promoting immune tolerance and anergy, the actual role of corresponding $CD8^+$ T cells in immune suppression and in the production of immunoregulatory cytokines is less understood and clearly deserves more studies.

Investigating immunopathological events during murine AE using cytokine detection, Ma et al. (2014) showed that IL-17 expression occurred in hepatic cells at 1 month postinfection (early stage infection), reached a maximum at 3 months postinfection (medium stage) and then decreased gradually (late stage). Compared with the uninfected control, levels of the cytokines IL-17, TGF-β1, IL-6, IFN-γ and IL-4 exhibited different dynamic patterns upon AE development. Conclusively, Th17 cells appear to play an important role by secreting IL-17, which may be involved in the Th1/Th2 cell imbalance developing during AE, and Th17 cells appear to be associated with immunopathology in murine AE. Treg/Th17 imbalance (Pang et al., 2014) was observed at the middle and even more at the late stage of *E. multilocularis* infection; results suggest that it may be regulated by the TGF-ββ/Smad signaling pathway (Pang et al., 2014). The subtle interplay of Treg and Th17 subsets with the various components of the TGF-β/Smad pathway in regulating immune tolerance and tissue inflammation in AE, thus facilitating the long-term survival of *E. multilocularis* in the host still deserves further studies.

In addition to cytokines, periparasitic granuloma formation is associated with different chemokines (Sadek et al., 1998; Wang et al., 2014a,b), which represent a family of molecules whose presumed primary function is to direct cellular movement and functional activation of primary effector cells (such as NK cells, polymorphonuclear cells and monocytes/MØs). Recent results on the expression of chemokines in the liver as well as in the periparasitic infiltrate in experimentally infected mice confirmed the importance of these molecules to maintain the granulomatous infiltrate at the proximity of the metacestode (Wang et al., 2014a,b). The course of Th1-related chemokines appeared 'complementary'; CXCL9 was more expressed when CXCL10 was less expressed, and vice versa, with a 'mirror' image, as previously described for IL-1 and IL-6. This may indicate some balance to ensure lymphocyte occurrence and persistence in the lesions. Th2-related

chemokines were also permanently expressed: expression of CCL12 and CCL17 followed the course of IL-4, and CCL8 followed the course of IL-5. Such changes in chemokine release may prevent pathogenic inflammation at the late stage of infection (Wang et al., 2014a,b). In addition, microarray gene expression analyses revealed a hyperexpression of RANTES (CCL5) (Wang et al., 2014a,b), a chemoattractant for Th1 cells, eosinophils and basophils (Mejri and Gottstein, 2009). This finding suggests that this chemokine is also secreted by cells of the granuloma at the early stage (8−30 days) when IL-12, IFN-γ and IL-17 secretions are at their maximum (Wang et al., 2014a,b).

CE: Experimental intraperitoneal infection of mice with *E. granulosus* protoscoleces showed a substantial local increase of $CD4^+$ T lymphocytes including Foxp3-expressing $CD4^+CD25^+$ T cells (Tregs) (Mourgia-Ettlin et al., 2011b). Initial studies on the relatively early immune response in *E. granulosus*-infected mice showed a defined Th2-type systemic cytokine profile, suggesting that immune polarization is an early event (Dematteis et al., 1999), which may actually help to prevent antiparasite resistance, such as it occurs in most intermediate hosts, including in the group of humans who do not develop disease. Experimental intraperitoneal infection of mice with *E. granulosus* protoscoleces showed an early predominant induction of Th1-type cytokines (IFN-γ, IL-2 and IL-15), followed by a shift towards a Th2-type profile (IL-4, IL-5, IL-6, IL-10 and IL-13) (Mourglia-Ettlin et al., 2011a,b). Pan et al. (2013) observed that T cells were activated following infection in BALB/c mice, but the significant increase of immunosuppressive cells such as myeloid-derived suppressor cells (MDSC) and Treg cells could inhibit T cell response to *E. granulosus* antigens. These immunosuppressive cells may play a key role in the downregulation of the immune response during long-term parasitic infection.

Regarding cellular immunity in these experiments, upregulation of activation markers CD69, CD44, CD40L, and downregulation of CD62L were observed in $CD4^+$ and $CD8^+$ T cells, and $CD25^+$/FoxP3−Tregs increased significantly over the course of infection (Pan et al., 2013). Mourga-Ettlin et al. (2016) reported recently that BALB/c and C57Bl/6 mice are high- and low-susceptible strains, respectively, to experimental infection with *E. granulosus*. Principal components analysis (PCA) clustered C57Bl/6 mice by their early mixed IL-5/TNF-α responses and less intense expression of Th2-type cytokines. With regard to the peritoneal cell composition, they exhibited lower eosinophils and higher numbers of MØs and B cells. Functional studies showed that peritoneal cells from infected C57Bl/6 mice

displayed greater antiparasite activities, in accordance with higher rates of NO production and more efficient ADCC responses. The authors concluded that moderate Th2 responses and active cellular mechanisms are key determinants in murine resistance to *E. granulosus* infection.

3. OVERVIEW ON IMMUNOLOGICAL ASPECTS ON HUMAN ALVEOLAR ECHINOCOCCOSIS AND CYSTIC ECHINOCOCCOSIS

3.1 Innate immunity

Basically, a successful larval infection with *E. multilocularis* starts with the rapid synthesis of the LL by the GL. LL is composed of mucins bearing defined GA lactose-rich carbohydrates and accompanied by calcium inositol hexakisphosphate deposits, as recently reviewed by Diaz et al. (2011). Further studies on the *Echinococcus* LL were made possible upon complete genomes and some RNAseq data now available for both *E. multilocularis* and *E. granulosus* (Diaz et al., 2015). These findings revealed that the *E. multilocularis* LL is probably formed by a single type of mucin backbone, while a second apomucin subfamily additionally contributes to the *E. granulosus* LL. Previously, suspected differences between *E. granulosus* and *E. multilocularis* in mucin glycan size have been confirmed and pinned down to the virtual absence of Galβ1—3 chains in *E. multilocularis*. The LL protects the GL from, e.g., nitric oxide produced by host's periparasitic MØs or DCs, and putatively also other innate effector mechanisms, and also prevents — to a certain extent — protective specific immune recognition by surrounding T cells. Nevertheless, the early oncospheral phase of infection appears to resist and will survive nonspecific innate immune reactions in AE patients, and the subsequently LL-protected metacestode vesicle/tissue embodies a structurally different type of antigen that the host has to face. We do not have any information about individuals where early innate immunity has been able to efficiently eliminate developing oncospheres. Nobody so far has successfully assessed *E. multilocularis* exposed populations for the presence of stage-specific antioncospheral antibodies: from such studies the resulting data on seroprevalence could have indicated the proportion of persons demonstrating early stage resistance to infection. Here we know that active immunization of noninfected intermediate hosts with oncospheral antigens such as Eg95 or Em95 can effectively protect the vaccinated hosts from primary peroral infections with *Echinococcus* eggs (Heath et al., 2012a,b).

3.2 The role of macrophages/Kupffer cells

The intrahepatic localization of developing *Echinococcus* vesicles inevitably leads to a confrontation between parasite surface molecules, represented by the glycans exposed on the surface of the parasite LL, the parasite metabolites released by these vesicles (so far largely uncharacterized) and host effector cells such as MØs/Kupffer cells and subsequently DC and respectively involved T cells. Decoding of *Echinococcus*-derived molecules by the innate immune system predirects the type of response subsequently elicited by the adaptive immune system. Hsu et al. (2013) recently reported that the mouse KCR(CLEC4F) binds to *E. granulosus* LL mucins and to *E. multilocularis* LL as well. These findings highlight the ancestral adaptations of *Echinococcus* spp. to rodents as intermediate hosts and to the liver as infection site. The KCR particularly also bound to characteristic LL carbohydrate motifs ending in Gal1-4Gal-1-3 or Gal1-4Gal-1-4GlcNAc (Hsu et al., 2013). Furthermore, phagocyte-specific S100 proteins were abundantly found in the pericystic area of infection (Basika et al., 2012), and the contribution of these proteins to the periparasitic inflammatory responses could putatively control the cyst size increase. How monocytes/MØs/Kupffer cells effectively contribute to the immune response during the acute and chronic stage of infection has not been elucidated yet in humans, affected either by CE or by AE. In particular, the polarization of monocyte-derived MØs in classically activated (IFN-γ-dependent) M1 cells or alternatively activated (IL-4/IL-13) M2 cells, which mirrors the Th1/Th2 polarization of T cells, has been documented to contribute differentially to the fibrotic process in parasitic diseases (Beschin et al., 2013), but this has not yet been addressed in AE nor in CE. On the other hand, a specific role for the so-called 'epithelioid cells', cells of the MØ lineage that are lined along the LL on the host's side in AE lesions in rodents as well as in humans has received little attention and should certainly be studied in depth. Immunosuppressive properties, associated with abundant release of IL-10, have been attributed to such cells in other diseases (Feng et al., 2014). Our knowledge of the characteristics of these cells in AE only includes their CD11b−, $CD25^+$ phenotype (Bresson-Hadni et al., 1994) and their strong expression of MICA/B, the stress-induced MHC class I chain–related molecules, which play a role in innate immunity and serve as ligands to NKG2D, an important activation receptor for NK cells and $CD8^+$ T cells (Zhang et al., 2008). The actual role of MICA/B overexpression in periparasitic epithelioid cells is however unknown: the lack of significant NK or

$CD8^+$ T cell cytotoxicity in AE seems to be due to the absence of expression of NKG2D despite the presence of its ligand MICA/B (Zhang et al., 2008).

3.3 The role of dendritic cells

AE: DCs represent key professional APCs that direct T cells in response to pathogen recognition. The marked periparasitic granulomatous infiltration found in hepatic AE indicates an intense parasite–host interaction. Host cellular immune responses appear crucial in the control of the metacestode growth kinetics, as well as its respective failure to control parasite growth, and most of such immune reactions are primarily triggered via DC activities. A first indication that DC function appears peculiar in human AE was provided by Jenne et al. (2001) who showed that a crude Em antigen failed to yield DC maturation but was nevertheless able to induce autologous $CD8^+$ T cell proliferation. Bellanger et al. (2015) demonstrated that *E. multilocularis* vesicle fluid antigen exerts a downregulating effect on the expression of costimulatory molecules (CD80, CD86 and CD83) of human monocyte-derived DCs and simultaneously an increase of TGF-β production. Nono et al. (2012) showed that after preincubation with either *E. multilocularis* primary cells or metacestode vesicles, DCs showed an impaired ability to be activated by the TLR ligand LPS. While neither primary cells nor metacestode vesicles induced the secretion of proinflammatory IL-12p70, the production of immunosuppressive IL-10 was elevated in response to primary cell E/S products (Nono et al., 2012).

CE: An established fluid-filled hydatid cyst can obviously escape destruction by the host immune response for long periods, which raises the question of how the parasite can evade host immune effector mechanisms. Kanan and Chain (2006) showed that *E. granulosus* metabolites stimulated the release of prostaglandin E2 (PGE2) and IL-6 upon incubation with human adherent peripheral blood monocytes (PBMCs) cultured in GM-CSF/IL-4. Concomitantly, the same incubation impaired the ability of these cells to secrete IL-12, IL-6 or PGE2 in response to LPS stimulation (Kanan and Chain, 2006). Conclusively, the parasite appears to actively divert the host immune system towards anergy and antiinflammatory pathways. Using *E. granulosus* metabolites plus the purified antigen B (AgB), Riganò et al. (2007) demonstrated that both these antigens exhibited an immunomodulatory potential on human DCs. During monocyte differentiation the Gabs downmodulated CD1a (the CD1 proteins mediate the presentation of primarily lipid and glycolipid antigens of self or microbial origin to T cells) and upregulated costimulatory CD86 expression. Furthermore,

AgB reduced the production of interleukin-12p70 (IL-12p70) and of TNF-α in LPS-stimulated DCs, AgB also induced IL-1 receptor-associated kinase phosphorylation and activated nuclear factor-kappaB (NF-kappaB), suggesting that TLRs could participate in *E. granulosus*-stimulated DC maturation. These results suggest that E. *granulosus* escapes host immunosurveillance by interfering with monocyte differentiation and additionally by modulating DC maturation (Riganò et al., 2007).

3.4 Acquired immunity: the role of different Th cell types (focusing on Tregs and Th17), including cytokines/chemokines

AE: Human patients suffering from chronic AE exhibit a rather Th2-dominated immunity associated with an increased susceptibility to disease. In contrast, a Th1-biased immune response induces protective immunity, which may even lead to aborted forms of infection (Rausch et al., 1987).

Earlier studies already documented that the switch from an initial Th1-orientation towards a rather Th2-type immune response was associated with the chronic and progressive course of disease and could thus be important for the lack of clearance of infection (Vuitton, 2003). The conventional immunocompetent but still susceptible AE patient exhibits – at a disease stage – usually a rather Th2-associated cytokine profile that encompasses high production of IL-4, IL-5 and IL-10 (Dreweck et al., 1999). IL-5 appeared as one of the predominant cytokines expressed by PBMCs in AE patients (Sturm et al., 1995; Godot et al., 1997), and Th2-type IL-13, IL-5 and IL-10 were enhanced in severely ill AE patients and in patients with genetic characteristics of susceptibility to *E. multilocularis* infection (such as HLA-DR3 DQ2) (Jenne et al., 1997; Wellinghausen et al., 1999; Godot et al., 2000a, 2000b), while *E. multilocularis* antigen-induced IFN-γ and spontaneous IL-12 production were decreased (Schmid et al., 1995; Hübner et al., 2006). At a relatively early stage of infection, elevated transcription levels of proinflammatory cytokines, e.g., IL-1β, IL-6 and TNF-α, as well as Th1 cytokines, i.e., IL-12 and IFN-γ, are characteristic. With increasing severity and chronicity of AE, the patient's immunity gets oriented towards Th2, including elevated IL-13, IL-4, IL-5 and IL-10 (Godot et al., 2000a,b; Vuitton et al., 2006). A further important downregulating cytokine that appears is TGF-β, which is prominent in the periparasitic infiltrate that surrounds the lesions in the liver of AE patients (Zhang et al., 2008). Other studies on the immunopathology of AE revealed that $CD4^{+}CD25^{+}$ Treg cells played a critical role in human AE by blunting immune responses to

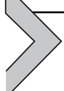
specific antigens, or by suppressing the secretion of proinflammatory cytokines, especially through IL-10 and TGF-β release (Hübner et al., 2006). Kocherscheidt et al. (2008) showed that the production of CC and CXC chemokines that associate with inflammation (MIP-1 alpha/CCL3, MIP-1 beta/CCL4, RANTES/CCL5 and GRO-alpha/CXCL1) were constitutively larger in AE patients than in controls; this was independent of the disease status (progressive, stable or cured AE). The fact that *E. multilocularis* metacestodes selectively suppressed cellular chemokine production in AE patients may constitute an immune escape mechanism, which reduces inflammatory host responses, prevents tissue destruction and organ damage but may also facilitate parasite persistence (Mejri et al., 2009).

Recently, the discovery of the IL-17 cytokine family has added a new dimension to the balance of inflammation and tolerance during parasitic infections. A recent study involving human AE patients showed that increased IL-17A expression was associated with protection, while upregulation of IL-17F expression might contribute to both protection and pathogenesis (Lechner et al., 2012). The decreased EmAg-specific IL-17F and IL-17RA production in AE patients with active *E. multilocularis* infection indicated a parasite-induced unresponsiveness; such cellular anergy may facilitate survival of the parasite in its host (Lechner et al., 2012). A remaining question concerns the role of TGF-β in the context of Th17 cell or Treg cells or both cell-type promotion. While TGF-β alone supports Treg cell expansion, TGF-β together with IL-6 promotes Th17 expansion. A careful assessment of IL-6 expression levels may help to elucidate this open question, and thus the putatively differential role of Th17 and Treg cells in susceptibility versus resistance to AE (Gottstein et al., 2015; Wang and Gottstein, 2016).

Immunogenic traits conferring resistance, susceptibility or associate with AE disease severity were suggested to be MHC-associated, here HLA-DRB1*11 might confer protection against AE and HLA-DQB1*02 may indicate a risk for progressive disease development (Eiermann et al., 1998). The HLA characteristics of the host, notably HLA B8, DR3, DQ2 haplotypes, can influence immune-mediated mechanisms, and the course of AE in humans, and specific antigenic components of *E. multilocularis* could contribute to the preferential Th2-type cytokine production favoured by the genetic background of the host (Godot et al., 2000b).

CE: In CE, crude sheep hydatid fluid elicits both Th1 and Th2 cell activation: globally, like in AE, the Th2 response benefits the parasite, whereas the Th1 response benefits the host (Riganò et al., 1995a,b; Riganò et al., 1999a,b). Thus, the characterization of parasite-derived immunoregulatory

molecules associated with Th1 or Th2 polarization is an important prerequisite for identifying the basis of resistance or susceptibility. Clearly, the Th1/Th2 imbalance plays an important role in controlling the immunopathogenesis of CE. Piccoli et al. (2012) studied the correlation between CE stage and selected Th1 versus Th2 serum cytokine profiles and found that IL-4 and IL-13 were associated with the cyst stage. A review of Siracusano et al. (2012a,b) concluded that patients who responded to chemotherapy produced high amounts of IFN-γ, whereas nonresponders produced predominantly IL-4 and IL-10. Patients who did not respond well to therapy weakly expressed IL-4 mRNA before therapy, and strongly thereafter, patients who responded to therapy expressed higher IFN-γ and TNF-α mRNA levels than patients who did not (Riganò et al., 1999a,b). The Th17/Treg functional imbalance exists also in patients with CE and plays a role in immune tolerance and the progression of the disease. The constant release of some active molecules by *E. granulosus* into the circulation during the course of chronic infection could actively modulate the host's immune system and shift the Th17/Treg balance to the Treg-dominant suppressive immune response via increased secretion of suppressive cytokines IL-10 and TGF-β1, hence inhibiting the inflammatory response induced by Th17 cells (Tuxun at al. 2012).



4. IMMUNOLOGY AND IMMUNOREGULATION OF SUSCEPTIBILITY/MORBIDITY IN ALVEOLAR ECHINOCOCCOSIS AND CYSTIC ECHINOCOCCOSIS

4.1 Immunomodulation that leads to alveolar echinococcosis /disease

For immunocompetent individuals, where *E. multilocularis* infection leads to disease (AE), the metacestode proliferation is very slow and appears to be partially controlled by host immunity. Human patients suffering from chronic AE present a mixed Th1/Th2 profile associated with the expression of proinflammatory cytokines in the periparasitic granuloma, and this mitigated inflammatory process appears to succeed in restricting the parasite growth upon formation of fibrosis and necrosis (Vuitton et al., 1989; Bresson-Hadni et al., 1990). Fibrosis is a hallmark in AE, gradually leading to a disappearance of the liver parenchyma and to the death of the metacestode, with vesicles embedded in an acellular tissue composed nearly entirely of cross-linked collagens (Vuitton et al., 1986). The diffusion of the fibrotic process even far from the parasitic lesions strongly suggests a major role for

cytokines in collagen synthesis. They may also be involved in cross-linking the collagen bundles in humans (Vuitton et al., 1986; Ricard-Blum et al., 1992, 1996). TGF-β, present in the cell infiltrate surrounding the parasitic lesions, in addition to its role in maintaining tolerance, is likely involved in the development of fibrosis in AE. The parasite itself could also be involved in the collagen cross-linking process, since a transglutaminase of parasitic origin has been shown to be strongly expressed in and at the border of the parasitic vesicles and is able to efficiently cross-link collagens of human origin in vitro (Grenard et al., 2001). Fibrosis, in addition to the LL, could be responsible for the protection of the parasite against any contact with both cytotoxic and antibody-secreting cells of the host and vice versa. It may explain the low rate of anaphylactic symptoms in patients with AE (Vuitton et al., 1988; Bresson-Hadni et al., 2000): the extremely fibrotic lesions of AE cannot rupture, and the echinococcal fluid may well be never in contact with mast cell-bound IgE, despite their constant presence, which could be demonstrated in vitro (Vuitton et al., 1988). In fact allergic symptoms rarely occur in patients with AE, they are observed only while parasitic cells migrate to other organs than the liver and are eventually leading to metastases, especially through pulmonary embolism (Bresson-Hadni et al., 2000). However, fibrosis is also the main cause for bile duct and vessel obstructions and thus, the pathophysiological background of chronic cholestasis, angiocholitis, portal hypertension, Budd–Chiari syndrome and/or vena cava obstruction (Bresson-Hadni et al., 2000).

Regarding the type of immune response at stake, locally close to the parasitic vesicles or in the circulation, since the 1980–90s explorative studies had clearly indicated that the cellular pathway selected during the course of infection strongly determines the clinical outcome of AE. A biased $CD4^+/CD8^+$ T cell ratio was observed in 'susceptible' patients with a severe and progressing disease and was determined by a marked increase of the $CD4^+/CD8^+$ ratio, mainly due to a decreased number of CD8 T cells among peripheral T lymphocytes, and the predominance of $CD8^+$ T lymphocytes within the periparasitic granuloma (Vuitton et al., 1989). In patients with chronic AE, long after the initiation of the disease, the generation of memory Th1 $CD4^+$ T cells was shown to be impaired (Manfras et al., 2004). In patients with a chronically progressing/severe AE, peripheral $CD8^+$ T cells have been shown to produce Th2 cytokines as well as IL-10 and TGF-β (Godot et al., 1997; Kilwinski et al., 1999; Zhang et al., 2008). Other immune effector cells, including NK- and/or T cell-dependent cytotoxic mechanisms, may be impaired by the cytokines

secreted abundantly in the periparasitic immune cell infiltrate and/or through cell–cell interaction mechanisms. The quasi-absence of NK cells and the inhibition of the expression of the costimulatory receptor NKG2D at the surface of CD8 T cells in the periparasitic granuloma have been shown in patients with AE (Zhang et al., 2008). Despite the presence of its ligand MICA/B at the surface of hepatic cells, epithelioid cells and the parasite GL itself, cytotoxicity of CD8 T cells might thus be severely impaired (Zhang et al., 2008). The lack of expression of NKG2D on $CD8^{+}$ T cells was not related to the presence of the soluble form of MICA/B, since this soluble form could not be detected in patients' sera; this absence could be due to the inhibitory effect of TGF-β, which is massively expressed by the lymphocytes surrounding parasitic vesicles (Eisele et al., 2006). In general, the nature and kind of the granuloma developed at the contact of the LL appears to be crucial in determining the potential of parasite proliferation during infection and disease. In addition to TGF-ββ, in the 'permissive' AE patient who develops chronic disease, this process encompasses a high production of IL-4, IL-5 and IL-10 (Dreweck et al., 1999). IL-10 was actually found to be abundant in the periparasitic granuloma surrounding the parasite (Harraga et al., 2003). Upregulation of IL-17F expression was claimed to contribute to pathogenesis as well (Lechner et al., 2012). There is evidence that TGF-β1, besides modulating immune tolerance, is involved in the synthesis of procollagen and other extracellular matrix components (Bartram et al., 2004; Higashiyama et al., 2007), it may thus play an important role in the pathogenesis of liver fibrosis that helps control parasite proliferation.

AE patients exhibiting an advanced stage of disease have repeatedly been subjected to liver transplantation as a putatively curative treatment option. Observations in transplanted patients, who received immunosuppressive agents to prevent liver rejection, confirmed the increased susceptibility to *E. multilocularis* growth in humans upon impaired immune responsiveness. Increased susceptibility was evidenced by a rapid increase in size of lung metastases, the development of brain metastases, late reinvasion of the transplanted liver by parasitic cell remnants and even early reinvasion of the transplanted liver from a spleen metastasis (Bresson-Hadni et al., 1999; Koch et al., 2003).

Individuals who get infected with *E. multilocularis* and who suffer from an impaired immunity, such as caused by AIDS, other immunodeficiencies or immunosuppressing immunotherapy (following organ transplantation or to treat malignancies or chronic inflammatory disorders), the metacestode

proliferation appears uncontrolled, leading to a very rapidly progressing disease status (Chauchet et al., 2014). On the other hand, it appears that downregulation of immunological side arms such as IgE-mediated allergy, e.g., via omalizumab treatment seems not to affect the proliferative growth of *E. multilocularis* (Skiepko et al., 2013), which can be explained by the fact that, despite a high production of IL-5 (Sturm et al., 1995; Godot et al., 1997), a cytokine well known to mobilize and activate eosinophils (Clutterbuck et al., 1989), *E. multilocularis* appears not to be a typical eosinophil-sensitive helminth, may be because the parasite actively downregulates eosinophil attraction (Mejri and Gottstein, 2009).

4.2 Immunomodulation that leads to CE/disease

Echinococcus granulosus can use two mechanisms to subvert the host immune response: passive escape, in which the parasite, by developing into a hydatid cyst, avoids the damaging effects of an immune response, and immunomodulation through which the parasite actively interacts with the host immune system to reduce the impact of host response (Siracusano et al., 2009a,b). In CE, LL represents a first barrier against host's immune attack; in addition, it is bound by a host-produced fibrous adventitious layer ('adventitia' or 'capsule'), which plays a role in the protection from host immune attack. The capsule is the product of a three-layered host cellular inflammatory-type response initiated in the early stages of postoncospheral development by infiltration of eosinophils, fibroblasts and mesothelial cells; the precise immunological reactions involved in the formation of the adventitia, which seems crucial to limit the cyst and protect the parasite, are poorly known and certainly need further evaluations.

Most parasites have evolved additional strategies for immune evasion that include antigenic variation, shedding of surface protein, protease production, active modulation including immunosuppression, skewing of the Th1/Th2 cytokine profile, molecular masking and mimicry, T cell suppression and modulation and inhibition of effector cell chemotaxis (Zhang et al., 2008). Recent studies demonstrated that *E. granulosus* secretes several molecules present in protoscoleces and in hydatid fluid that directly can modulate the immune responses thus altering the cytokine balance towards Th2 and favouring immune evasion and perpetuating parasite survival in the host (Riganò et al., 2004; Siracusano et al., 2008). These molecules interfere with antigen presentation, cell proliferation and activation, antibody production; they may cause cell death and they stimulate regulatory responses. The clinically observed immunological consequences of CE (both related

and unrelated to cyst rupture) arise from (1) immunosuppression, (2) complications associated with circulating immunological complexes and in particular (3) acute hypersensitivity reactions. The Th2 polarization induced by the parasite leads to hypersensitivity reactions that could vary widely, from benign urticaria and short episodes of shaking chills or fever, or both events, to potentially fatal bronchial spasms, angioneurotic oedema and anaphylactic shock. The latter occurs most frequently after the accidental rupture of the hydatid cyst or during surgery (Pawlowski, 1997). An understanding of the complex pathogenic mechanisms leading to an allergic reaction in CE requires information about the structure and function of allergenic proteins, namely antigens recognized by IgE. Several *E. granulosus* allergens have been isolated and characterized such as EgEF-1 β/δ, EA21, Eg2HSP70, EgTeg and Eg19 that play a critical role in the allergic reactions manifested by patients (Siracusano et al., 2012a,b).

5. IMMUNOLOGY AND IMMUNOREGULATION OF RESISTANCE TO ALVEOLAR ECHINOCOCCOSIS AND CYSTIC ECHINOCOCCOSIS

5.1 Immunomodulation that leads to inactivation/dying out/abortion of alveolar echinococcosis

Based on epidemiological investigations, there is accumulating evidence that a large part of humans exposed to infection with *E. multilocularis* appears resistant to disease development, as indicated either by parasite-specific seroconversion or by presenting intrahepatic 'died-out' or 'aborted' lesions that are accidentally detected following hepatic imaging procedures (Rausch et al., 1987; Bresson-Hadni et al., 1994; Romig et al., 1999; Gottstein et al., 2001; Bartholomot et al., 2002). The pathological key characteristic of AE is fibrosis, which destroys the liver parenchyma and consequently is responsible for most of the clinical manifestations. However, the embedding of the metacestode vesicles/tissue in heavily cross-linked collagens can also lead to metacestode death. Extensive extracellular matrix cross-linking is initiated by a parasite-associated transglutaminase (Grenard et al., 2001). In addition, soluble mediators directly or indirectly diffused by the parasite appear to mediate fibrogenesis in a more general manner, as fibrosis arises in hepatic zones far from the metacestode lesion (Ricard-Blum et al., 1992, 1996). Recent studies on the role of TGF-β in AE host–parasite relationship have contributed to give mechanistic support to these findings (Zhang et al., 2008).

Responses characteristics for AE resistance have not been fully elucidated so far. A trend towards resistance against disease was principally associated with Th1 cytokine profiles including IFN-α (Godot et al., 2003) and IL-12 (Emery et al., 1998) as initiating cytokines, and IFN-γ (Liance et al., 1998) and TNF-α (Amiot et al., 1999; Shi et al., 2004) as effector cytokines.

Furthermore, AE patients with aborted lesions presented lower secretion levels of IL-10 and Th-2 cytokines by PBMCs than patients with a progressing disease (Godot et al., 2003); another study suggested that progression of lesions could be associated with genetic determinants of the immune response, such as the presence of the HLA-B8, DR3, DQ2 haplotype (Godot et al., 2000b) and biases in the polymorphism of TAP1 and 2 genes (Zhang et al., 2003). Other cytokines and chemokines secreted by the periparasitic immune cells also seem different in patients with regression versus progression of the disease (Kocherscheidt et al., 2008; Huang et al., 2014). Kocherscheidt et al. (2008) studied chemokine responses in AE patients at different stages of infection (progressive, stable or cured AE). The production of CC and CXC chemokines was associated with inflammation (MIP-1 alpha/CCL3, MIP-1 beta/CCL4, RANTES/CCL5 and GRO-alpha/CXCL1) and it appeared constitutively larger in all groups of AE patients when compared to controls (Kocherscheidt et al., 2008). This disparate cellular responsiveness to viable *E. multilocularis* vesicles was observed in all groups of AE patients; cluster 1 (GRO-alpha/CXCL1, MCP-3/CCL7, MCP-4/CCL13, TARC/CCL17, LARC/CCL20) and cluster 2 chemokines (PARC/CCL18, MDC/CCL22, MIG/CXCL9) were downregulated, while cluster 3 chemokines (MIP-1 alpha/CCL3, MIP-1 beta/CCL4, RANTES/CCL5) appeared upregulated (Kocherscheidt et al., 2008). Furthermore, proinflammatory IL-31 and IL-33 were depressed in all AE patients, while regulatory IL-27 and CCL11, CCL24, CCL26 became more elevated during disease progression. In patients with cured AE, however, the production of several inflammatory chemokines persisted, and this could have been induced by residual *E. multilocularis* metacestode lesions, which continuously stimulated the production of these inflammatory immune mediators.

5.2 Immunomodulation that leads to inactivation/dying out/abortion of cystic echinococcosis

Evidence suggest that the intermediate hosts respond sequentially (time-dependent) and, to some extent, also stage specifically to antigenic stimuli of the invading oncosphere, the metacestode in transformation from the

oncosphere, and finally, the mature metacestode (larvae) (Zhang et al., 2008). The oncospheres hatch and become activated in the small intestine when a suitable intermediate host ingests *Echinococcus* eggs. Lytic secretions of the oncosphere facilitate its passage through the intestinal mucosa and into the host circulatory system (venous and lymphatic) through which they are distributed to the liver, lungs and other sites where postoncospheral development continues. Within a few days, oncospheres reach the preferred site where cystic development begins. Since the oncosphere is known to be associated with the protective immune response, understanding the mechanisms of action of protective antibodies is of fundamental importance in developing highly effective vaccine against *E. granulosus* (Zhang, 2008b). The protective effect, at this developmental stage, of a vaccine based on the recombinant oncosphere protein, Eg95, was demonstrated and Eg95 is produced and routinely used for prevention of infection in the parasite's natural animal intermediate hosts (Lightowlers et al., 1996; Gauci et al., 2005). In a similar context, it would be interesting to know if a natural *E. granulosus* infection, which includes the establishment of an immune response unable to eliminate the parasite, could exhibit an appropriate anti-oncospheral immune response, or at least a response against parasite antigens that are also shared by oncospheres, thus yield a naturally acquired protection against reinfection by *E. granulosus*. In this context, first indications about the existence and function of 'concomitant' immunity have already been provided, based upon, respectively, explorative experiments (Heath et al., 1981; Dempster et al., 1992; Rogan et al., 1992).

In few cases where parasite abortion (dying out of a CE cyst) has been documented, information on the kind and pattern of 'protective' immunity is of special interest. In 2001, the WHO Informal Working group on Echinococcosis proposed an international classification of hepatic cysts based on US morphology correlated to the activity of the disease (WHO, 2003). Following the WHO classification, hepatic hydatid cysts are grouped into five major cyst types, CE1−CE5, characterized by the appearance of the cyst contents and wall. Type CE1 (unilocular, simple cysts) and type CE2 (multivesicular, multiseptated cysts) are considered as active since they likely contain viable protoscoleces. Type CE3 (unilocular cysts with detachment of laminated membrane or multiseptated cysts with partial hyperechoic content) are considered as transitional and might represent the beginning of cyst degeneration. Type CE4 (heterogeneous or hyperechoic degenerative contents) and type CE5 (calcified cysts) are considered inactive (Brunetti et al., 2010). Typical immunological profiles characterizing CE4 cysts include

large leucocyte infiltrates and often elevated antibody levels (Rogan et al., 2015), whilst CE5 cysts are totally inactive with reduced antibody profiles (Tamarozzi et al., 2013). Furthermore, it has been claimed that specific serologic profiles are associated with cysts of the same ultrasonographic types (types CE3—4—5). Higher serum IgG1 and IgG3 have been observed in stable disease and higher IgG4 and IgE in progressive disease (Riganò et al., 2002). It is generally accepted that *Echinococcus* is unaffected by the humoral immune response during the developing stage and there are no detailed studies of immunological events associated with the degeneration of different types of cyst.

To note, genetic factors could contribute to the severity of CE (HLA-DR3, -DQ2) and predispose for the development of disease (HLA-DP 0401) (Kiper et al., 2010). During chronic infection and persistent immune responses, antigenic peptides degraded by proteasomes are secreted into the lumina of the rough endoplasmic reticulum through ATP-dependent pumps, the transporters of antigenic peptides (TAP1 and TAP2); these parasite-derived peptides may then become presented by MHC class I molecules on $CD8^+$ T lymphocytes, and TAP1-637 and TAP2-379 gene polymorphisms may associate with the development of CE.

6. STATUS OF IMMUNOTHERAPY AND VACCINATION AGAINST ALVEOLAR ECHINOCOCCOSIS AND CYSTIC ECHINOCOCCOSIS

6.1 Parasite antigens and metabolites

AE: Excretory/secretory (E/S) metabolic products of the *E. multilocularis* metacestode are among the key players for modulating host immune regulatory events. Functionally, they mainly focus onto downregulating the host immune response such as to promote metacestode survival. Downregulation of immunity may also include the suppression of immunopathological events, such as found in other diseases than AE. Consequently, one might speculate that those parasite metabolites that specifically abrogate certain immune parameters may also help to control excessive inflammatory responses specifically encountered in other noninfectious diseases such as inflammatory bowel conditions, multiple sclerosis, asthma and atopy. Application of defined parasite metabolites has been suggested as a possible treatment option for autoimmune and other inflammatory disorders in humans (Helmby, 2015). Proof of principle had already been achieved by earlier experiments, where an 'intestinal nematode worm treatment'

(*Trichuris suis* eggs) was successfully administered to patients suffering from ulcerative colitis or Crohn's disease (reviewed by Helmby, 2015). Subsequently, research focused on the identification of the functional metabolites found in various helminths so far. One of the best-characterized product so far is the ES-62 glycoprotein from the filarial nematode *Acanthocheilonema viteae*, which skews DCs towards promoting a Th2 orientation of cell-mediated immunity, whilst inhibiting Th1 and Th17 polarization. Since then, other metabolites from other helminths have been characterized, most of them belong to a family of immunomodulatory proteins, termed helminth defence molecules (HDMs), which are secreted by several medically important helminth species (Alvarado et al., 2015). These HDMs share biochemical and structural characteristics with mammalian cathelicidin-like host defence peptides (HDPs). Parasite HDMs block the activation of MØs via TLR 4 signaling, however HDMs are significantly less cytotoxic than HDPs. In another study, *A. viteae* cystatin was also found to block grass pollen-specific allergic responses in the lungs, in particular by inhibiting eosinophil infiltration and IL-5 and IL-13 cytokine levels (Danilowicz-Luebert et al., 2013). An overview on the potential of secretary products of helminths as immunomodulators is written by Harnett (2014).

Regarding *E. multilocularis*, experimental results suggest that the immunoregulation elaborated by parasite during infection (AE) may actually prolong the survival time of organ transplants in the same animal (Li et al., 2011). Immune modulatory effects of *E. multilocularis* metacestode in vitro culture supernatant, of Em vesicles and of vesicle fluid antigen were described, and the effect was, e.g., shown as a depressed release of proinflammatory IL-12 and TNF-α by PBMCs (Hübner et al., 2006). The production of IL-12 and TNF-α was reduced in AE patients, accompanied by an increased number of $CD4^{+}CD25^{+}$ Treg cells and a reduced release of the Th2-type chemokine CCL17/TARC. In parallel, production of the Th2-type chemokine CCL22/MDC was increased supporting that *E. multilocularis* would also generate proinflammatory immune responses. So far, an overall of various classes of molecules have been investigated to find biofunctional molecules with specific actions:

Glycosphingolipids: A neutral glycosphingolipid of *E. multilocularis* was found that can suppress human PBMCs proliferation following stimulation by phytohaemagglutinin (Persat et al., 1996).

Carbohydrates/glycans: Carbohydrate-rich components of the LL, such as Em2(G11) (Deplazes and Gottstein, 1991; Huelsmeier et al., 2002) and Em492 (Walker et al., 2004), as well as other parasite metabolites, may

express immunomodulatory effects (reviewed in Zheng, 2013); on the other hand, they appear very skilled in avoiding conventional immune recognition and thus elimination by host effector mechanisms. As an example, IgG response to the Em2(G11) antigen takes place independently of $\alpha\beta^{+}CD4^{+}$ T cells, and in the absence of interactions between CD40 and CD40 ligand (Dai et al., 2001). Em2(G11) does not induce maturation of DCs (Margos et al., 2010). Soluble Em492 (Walker et al., 2004) glycan antigen suppresses ConA and antigen-stimulated spleen cell proliferation and may thus avoid conventional immune recognition as well.

Glycoproteins/proteins: Metabolized *E. multilocularis* molecules such as novel mucin-type glycoforms, or protoscolex-associated proteins of 62, 70 and 90 kDa and several recombinant *E. multilocularis* proteins have all been published and discussed in view of their potential immunomodulatory functions (reviewed by Huelsmeier et al., 2002, and Zheng, 2013). Recently, it was showed that EmACT, a secreted metacestode activin, was able to induce expansion of host Treg cells, and thus appears to have an important role in immunomodulation (Nono et al., 2012). Another parasite factor named EmTIP, homologous to mammalian T cell immunomodulatory protein (TIP), was detected in secretory fractions of *E. multilocularis* primary cell cultures (Nono et al., 2014). EmTIP neutralization inhibited primary cell proliferation and the formation of metacestode vesicles in vitro, suggesting that this protein may be functionally important for the parasite development. EmTIP also evoked a strong release of IFN-γ by $CD4^{+}$ T cells, hence suggesting that the secretion of this factor could 'secondarily' induce a potentially protective Th1 response. The *E. multilocularis* phosphoglucose isomerase (EmPGI) (Stadelmann et al., 2010) is a glycolytic protein that is released into the vesicle fluid and stimulates growth of GL cells in vitro. However, EmPGI also stimulates endothelial cell proliferation, which may contribute to the support of metacestode proliferation by orchestrating the periparasitic host cell composition. *Echinococcus multilocularis* was shown to express cathepsin L-like cysteine proteases (EmCLP1 and EmCLP2) and to secrete these products into the periparasitic area (Sako et al., 2007). EmCLP1 and EmCLP2 are capable of degrading extracellular matrix proteins and may thus play a role in the invasive growth behaviour of the metacestode. Other cysteine proteases (EmCBP1 and EmCBP2) identified in *E. multilocularis* (Sako et al., 2011) were claimed to participate in immunomodulatory events, such

as inhibition of T cell proliferation, but this needs to be confirmed. Most of the parasite metabolites are claimed not only to be secreted externally by *E. multilocularis* vesicles but to accumulate also within the intravesicular fluid.

Other molecules: Larval stage metabolites coupled to genomic data (Brehm and Spiliotis, 2008; Brehm 2010) yielded information that a series of evolutionarily conserved signaling molecules are able to functionally interact with corresponding host cytokines. *Echinococcus multilocularis* expresses also a set of nuclear receptors, one of which (EmNHR1) cross-communicates with TGF-β signaling components (Förster et al., 2011).

Overall, immunoactive *E. multilocularis* metabolites represent not only interesting targets for immunotherapy of AE but they have also a high potential to be used for the treatment of autoimmune and/or inflammatory disorders (Pineda et al., 2014).

CE: As for AE, metabolic products from *E. granulosus* larval stages are supposed to play a fundamental role in the survival strategy of the parasite. Few studies have been carried out with the key stages, represented by either oncospheres or LL protected cysts. Most information was obtained so far by studying E/S products released by in vitro cultivated protoscoleces. An LC–MS/MS proteomic analysis of protoscolex E/S products yielded an identification of 32 proteins, including proteins already well identified as *Echinococcus* spp. antigens, such as EG19, P-29 and a calpain protease (Virginio et al., 2012). Among them, thioredoxin peroxidase and 14-3-3 proteins were postulated to be involved in evasion mechanisms adopted by the parasite to establish infection. Of the various proteins isolated from hydatid fluid and characterized, the principal *E. granulosus* immunomodulatory antigen is AgB (Riganò et al., 2007). Since it can modulate both innate and adaptive host immune responses, AgB plays a prominent role in the immunomodulatory mechanisms that *E. granulosus* uses to grow, progress and cause chronic disease. To survive in host tissues the parasite must be able to adapt metabolically to the host microenvironment and plentiful AgB in hydatid cyst fluid probably guarantees parasite survival (Siracusano et al., 2012a,b). AgB is a thermostable lipoprotein encoded by a multigene family having at least five gene loci (B1–B5), each consisting of several minor variants grouped into two clusters: EgAgB1/B3/B5 and EgAgB2/B4 (Fernández et al., 1996; Chemale et al., 2001). How can AgB modulate host immune response? It acts directly on innate and acquired host immunity. First, distinct studies

showed that the 12 kDa subunit of AgB is a serine protease inhibitor with strong chemoattractant activity and with the ability to inhibit human neutrophil chemotaxis (Sheperd et al., 1991; Riganò et al., 2001). AgB has been discussed to potentially act as an interference antigen allowing the released protoscoleces to develop into secondary cysts (Virginio et al., 2007). Patients' PBMC stimulated with AgB produced IL-4, IL-13 and low IFN-γ concentrations but did not produce IL-12. This Th2 polarization was more evident in patients with active disease, in whom the stimulus with AgB increased the imbalance observed in lymphocytes from patients with inactive disease (Riganò et al., 2004). Moreover, AgB modulates sentinel DC maturation, priming those cells to polarize lymphocytes into an exclusive Th2 response that benefits the parasite. If AgB encounters immature DCs, it suppresses IL-12p70 production by inducing the immunoregulatory cytokine IL-10. AgB reduces LPS-induced production of IL-12p70 but not of IL-6, providing further evidence that it actively modulates DC responsiveness in a manner favouring a Th2 outcome (Riganò et al., 2007). All these data suggest that AgB directly immunomodulates the host immune response by inhibiting polymorphonuclear cell chemotaxis and indirectly by skewing the Th1:Th2 cytokine ratio towards a preferentially Th2 polarization associated with chronic CE. In patients with CE, besides AgB, many other parasite molecules (such as EgTeg, and EgEF-1β/δ), can elicit Th2 production. All these antigens contribute to the immunomodulating properties of the chronic disease not only through their intrinsic ability but also by strengthening the generalized Th2 polarization previously established (Margutti et al., 1999; Ortona et al., 2001, 2005). Antigen 5 (Ag5) has been identified as a dominant component of cyst fluid of *E. granulosus* and is considered as a member of serine proteases family, which in other helminths, plays an important role in egg hatch and larva invasion. Ag5 is strongly expressed in the tegument of protoscolex, the embryonic membrane of eggs and at the surface of oncospheres; it is also weakly expressed in tegument of the adult. It may be anchored in the membrane by its myristoylation sites; these characteristics make it a candidate antigen for diagnosis and potentially for immunotherapy, both in intermediate and definitive hosts (Li et al., 2012a,b).

Helminth parasite glycoconjugates have important roles in driving cytokine response polarization (Okano et al., 1999; Terrazas et al., 2001). E4(+) (a glycoconjugate-enriched fraction from *E. granulosus* protoscolex) stimulated the secretion of a high concentration of IL-6, followed by

IL-10 and TNF-α by normal peritoneal B cells. Moreover, $E4^{+}$ triggers the production of IgM antibodies that bind to *E. granulosus* antigens and, through the activation of the complement system, could be part of the mechanism involved in the elimination of the protoscolex (Baz et al., 2008; Mourglia-Ettlin et al., 2011b). Wang et al. (2015a) studied the effects of *E. granulosus* (E/S) products on murine bone marrow-derived dendritic cells. Based on their experimental findings, the authors concluded that E/S of adult *E. granulosus* inhibited DC function, impaired the development of Th1 cells induced by CpG and induced $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ regulatory T cells in an IL-10-independent manner (Wang et al., 2015a).

6.2 Experimental immunotherapy or immunoprophylaxis

Immunotherapy/AE: Cytokines that promote differentiation of Th1 cells such as IL-12 (Emery et al., 1998) and IFN-α-2a (Liance et al., 1998; Godot et al., 2003) have been assessed for passive immunotherapy of AE in experimentally infected mice. Thus, in vivo treatment with recombinant IL-12 in mice with an established AE infection was shown to reduce the parasite burden as soon as 2 weeks after the end of treatment (Emery et al. 1998). Godot et al. (2003) investigated the effects of recombinant IFN-α-2a in *E. multilocularis*-infected mice. The study showed that 75% of IFN-α-2a-treated mice had no hepatic lesions, and half of these animals appeared fully protected. IFN-α-2a treatment down-regulated also the production of IL-10 and restored phagocytosis and oxidative metabolism of MØs (Godot et al., 2003). The effects of 1 or 5 micrograms of IFN-γ per day twice a week on murine AE were analyzed after 3 weeks of treatment. The treatment with 1 microgram transiently reduced the liver metacestode load, together with a slightly increased Th1-type T cell response (Liance et al., 1998), but so far no further development of this approach has been carried out. With regard to human AE, TNF inhibitor treatment was attempted once in a patient who suffered from rheumatoid arthritis and AE (Weiner et al., 2011). The *Echinococcus* P29 protein was initially described from *E. granulosus* as a promising diagnostic reagent for the follow-up of treated CE cases. Studies of the *p29* gene at inter- and intraspecies level (Boubaker et al., 2014) showed a high level of conservation (97% AA sequence identity) among different *Echinococcus* species/isolates, including *E. multilocularis*. Studies on immunological properties of bacterially expressed recombinant recEmP29 antigen (Boubaker et al., 2015) showed that postinfection immunotherapy with the recEmP29 resulted in a 53% reduction of parasite load as

compared to nontreated mice. Vaccination or treatment with recEmP29 was found to exhibit low ratios of IL-4/IFN-γ cytokine mRNA levels (Th2/Th1) and low IL-10 and IL-2 mRNA levels. These results suggested that anti-recEmP29 treatment results in an increased Th1-type immune response, which may be responsible for a partial restriction of parasite growth. This latter study provided one of the first evidence that active immunotherapy with appropriate parasite antigens may present a new alternative route for the control of AE.

Vaccines/AE: In murine AE, earlier experimental reports suggested that several antigenic compounds isolated from *E. multilocularis* may yield good protection against primary infection when used as a vaccine. Table 1A provides a list of different *E. multilocularis* antigens that have been used for vaccination trials in rodents, most of the challenge infections were done by infecting mice perorally with *E. multilocularis* eggs, which corresponds to natural infection way/mode of the parasite.

Siles-Lucas et al. (2003) identified and cloned a *14-3-3*-gene of *E. multilocularis*, which appeared to play a key role in basic cellular events related to cellular proliferation, including signal transduction, cell cycle control, cell differentiation, and cell survival. recEm14-3-3 was shown to protect mice against primary but not secondary AE (Siles-Lucas et al., 2003), yielding a protection rate of 97%, based upon the reduction of hepatic lesions recovered after peroral infection of mice with *E. multilocularis* eggs. Gauci et al. (2002) identified a cDNA-encoding antigen (designated EM95), determined the structure of the *em95* gene and demonstrated that the recombinant EM95 protein could be used to induce significant levels of protection against challenge infection with *E. multilocularis* eggs in mice (Gauci et al., 2002), overall protection rate was 83%. Wang et al. (2014a,b) cloned, out of the *em95* gene, three B and T cell-combined epitopes and expressed them in a prokaryotic PET32a vector. The resulting recombinant antigens rEm95-1 and rEm95-2 were then suggested to be used for the construction of high-valence vaccines and as targets for prevention of echinococcosis (Wang et al., 2014a,b), but so far the appropriate challenge experiments are still missing. In a similar context, Kouguchi et al. (2007) identified a cDNA clone, designated EMY162, which encoded a putatively secreted protein. EMY162 shares structural features with the EM95 antigen, for example, there was 31% amino acid sequence identity to EM95. A recombinant EMY162 antigen induced a significant level of host protection (74.3%) in experimental infection with *E. multilocularis* eggs in mice, indicating

Table 1 Vaccine candidates experimentally used to protect against challenge infection with *Echinococcus* sp.

Vaccine	Molecule(s)	Adjuvant	Protectivity	Host	Reference
A: Vaccine candidates for protecting against infection with Echinococcus multilocularis metacestodes (Challenge infection with parasite eggs)					
rEM95	Oncospheral penetration gland protein	Saponin	83%	Mouse	Gauci et al. (2002)
rEmy162	New secreted protein	Freund's c/i	74%	Mouse	Kouguchi et al. (2007)
rEm-TSP3	Tetraspanin 3	CpG OND	82%	Mouse	Dang et al. (2012)
rEm14-3-3	14-3-3-Protein	Saponin	96%	Mouse	Siles-Lucas et al. (2003)
rEm95-1, rEm95-2	Oncospheral penetration gland protein	Freund's c/i	(No challenge infection[a])	Rabbit	Wang et al. (2014a,b)
B: Vaccine candidates for protecting against infection with Echinococcus granulosus metacestodes (Challenge infection with parasite eggs)					
rEg95	Oncospheral penetration gland protein	QuilA	97%	Sheep	Lightowlers et al. (1996)
rEg95	Oncospheral penetration gland protein	QuilA	87—100%	Cattle	Heath et al. (2012)
rEgG1Y162	New secreted protein	rBCG	75%	Mouse	Ma et al. (2015)
rEg 14-3-3	14-3-3-Protein	Freund's c/i	85%	Mouse	Li et al. (2012a,b)
rEgGST	Recombinant *Echinococcus granulosus* glutathione S-transferase	Freund's c/i	89%	Mouse	Zhu et al. (2015)
	Myophillin	(Not declared)	69%	Mouse	Ma et al. (2012)
Multi-T cell epitopes	Proteins (EgGST, EgA31, Eg95, EgTrp and P14-3-3)	Freund's c/i	99%[b]	Mouse	Esmaelizad et al. (2013)
rEg-P29	'Diagnostic metacestode antigen'	Freund's c/i	96%[b]		Shi et al. (2009)

[a]Experiments were done out for primary antibody production, thus no challenge infection experiments were carried out so far to determine protectivity levels.
[b]Challenge infection with protoscoleces.

that EMY162 could be a candidate antigen with potential for use in a vaccine. Following bioinformatic prediction of epitopes in the Emy162 antigen of *E. multilocularis*, numerous distinct antigenic epitopes of Emy162 were identified. Five T cell- and seven B cell-dominant epitopes of the Emy162 antigen were revealed by the bioinformatic approach, which might be of use in the development of a dominant epitope vaccine (Li et al., 2013). Dang et al. (2009) evaluated seven tetraspanins of *E. multilocularis*, designated as TSP1—TSP7, for their protective potential against primary AE. The results indicated that recombinant tetraspanins have varying protective effects against primary AE and could be thus putatively used in vaccine development (Dang et al., 2009).

Immunotherapy/CE: Steers et al. (2001) showed that *E. granulosus* cysts incubated in vitro in the presence of nitric oxide (produced either from S-nitroso-N-acetylpenicillamine or IFN-γ-activated peritoneal murine MØs) underwent damage and death after 3 days, indicating that intact hydatid cysts could be susceptible to a Th1-driven MØ attack. A crude extract of the LL from cysts was found able to reduce the production of nitric oxide from activated MØs in vitro and in vivo and this may have been due to phagocytosis of LL fragments by the MØs (Steers et al., 2001). Conclusively, it appears that cysts may be susceptible to the effects of nitric oxide, but conversely the LL may be involved in downregulating detrimental nitric oxide production. The biological function of the LL of *E. granulosus* was studied by Noya et al. (2013) in view of its potential immunomodulatory capacity. The authors isolated mucin peptides from the *E. granulosus* LL and showed that they were capable of inducing an increase of activated NK cells in the spleen of immunized mice, a fact that was correlated with the capacity of spleen cells to mediate killing of tumour cells. The study demonstrated that the mucin peptides enhance LPS-induced maturation of DCs in vitro by increasing the production of IL-12p40p70 and IL-6 and that mucin-treated DCs may activate NK cells (Noya et al., 2013). The authors subsequently discussed the use of parasite-derived molecules such as mucins from *E. granulosus* in the fight against cancer.

Vaccines/CE: A list of different *E. granulosus* antigens that has been used for vaccination trials is presented in Table 1B. Vaccination against CE using a defined recombinant antigen, the oncospheral protein EG95, was shown to induce a protective response against hydatid infection in animal intermediate hosts of *E. granulosus* (Lightowlers et al., 1996), yielding overall protection rates of 97% in sheep (Lightowlers et al., 1996) and of

88–96% in cattle (Heath et al., 2012a,b). Subsequent studies on the amino acid substitutions of EG95 in the G6/G7 genotypes showed that there might be a potential affection of the antigenicity/efficacy of the EG95 recombinant vaccine against parasites of these latter genotypes (Chow et al., 2008). recEG95 induced a protective response against a challenge infection with *E. granulosus* with a similar efficacy in different other animal groups and species (Lightowlers et al., 1996; Heath el al., 2003; Barnes et al., 2009; Lightowlers et al., 1999). The prediction of T cell and B cell-combined epitope and tertiary structure of the Eg95 antigen of *E. granulosus* indicated that designing an antigen on the T- and B-combined epitopes would be an effective method in genetically engineered vaccine design, with potentially beneficial applications in CE prevention (Ma et al., 2013). Induction of protective Th1 immune responses against *E. granulosus* in mice by a multi-T cell epitope antigen based on five proteins (EgA31, EgTrp, EgGST, P14-3-3 and Eg95) showed the successful application of the predicted T cell epitope in designing a vaccine against *E. granulosus* in a mouse model (Esmaelizad et al., 2013). Li et al. (2012a) investigated the protective immunity potential of rEg14-3-3 in immunized mice. As the challenge was only with *E. granulosus* protoscoleces (protection rate of 84.5%), the practical challenge mode with parasite eggs is still needed to yield relevance in the context of CE control (Li et al., 2012a). Shi et al. (2009) cloned the gene, and subsequently expressed the antigen P-29 of *E. granulosus*, and used this recombinant antigen to vaccinate mice. Animals were challenged by intraperitoneal inoculation of *E. granulosus* protoscoleces, thus mimicking a secondary infection mode. Although the protection level obtained was high (96.6%), the biological meaning of these experiments, also in this case, appears not very relevant in the context of CE control (Shi et al., 2009).

As outlined earlier for *E. multilocularis*, also for *E. granulosus*, highly effective vaccines have been produced which are capable of preventing infections in its animal intermediate hosts. Application of vaccines at large scales, together with taeniacides in the parasites' definitive hosts, has already provided new opportunities for the control of CE and a reduction in its global burden in humans (Lightowlers, 2013). Conversely to *E. multilocularis*, molecular techniques have evidenced a significant genetic diversity for *E. granulosus* sensu lato, and various species and genotypes are now described, differing both genetically and in various biological aspects such as intermediate host preference (Lightowlers, 2013). But, as outlined earlier, efficiency of Eg95 appears not to be affected by this diversity. Nevertheless, at this time

there are still open questions and points that need further elucidation with regard to vaccination. For instance, there is no clear evidence of exactly how long immunity lasts in animals vaccinated with EG95. At least a 1-year-immunity has been demonstrated (Heath et al., 2003). So far, we do not know the possible differential effect of repeated reinfection upon a given infection pressure or of absence of reinfection if prevalence in the definitive host decreases, and if more booster injections are needed, etc. Ongoing larger-scale vaccination studies, however, should and will answer such questions.

Overall, within the genus *Echinococcus*, including here *E. multilocularis*, vaccination against primary infection appears to be a very promising approach to contribute to the control of infection. As for *E. granulosus*, the protection levels are very high, and virtually unique among helminthic infections. One reason might be found in the conserved oncospheral antigenicity at the early metacestode stage of these parasites. The oncosphere enters its nonimmune host in a very rapid way and reaches its final destination within minutes or hours, where it starts to rapidly maturate into a LL-protected proliferating stage. Obviously, innate immunity does not eliminate this early establishing oncosphere. It takes the host approximately 5–7 days to mount a parasite-specific immune response (including humoral and cellular components). During that time, the LL has been fully synthesized and provides a physical barrier against immune attack (Gottstein et al., 1992). Before being protected, the oncosphere seems to offer a large range of immune targets that allow inactivation or killing of the parasite, as shown by the large range of metabolized, membrane-bound and even cytoplasmatic proteins/antigens (e.g., 14-3-3) that have been used to successfully vaccinate mice. As some of them reach a protection level above 90%, a combined application of different antigens could synergize their effect and allow a maximum of protection. Summarized, the short window offered by the oncosphere that allows a relatively easy immunological clearance during the first phase of infection has made of *Echinococcus* one of the most promising helminth candidates for efficient protective immunization; this appears to hold true for the whole Taeniid family (Lightowlers et al., 2016).

Vaccines against adult stage *Echinococcus* sp.: In comparison with intermediate hosts of *Echinococcus*, limited and still controversial information is available regarding any immunology-based protective responses to *Echinococcus* infection in definitive hosts (Torgerson, 2009). Therefore, the authors of this chapter decided not to include this topic into their work. There

opinion is that there is much more basic experimental work to be performed before a solid presentation of facts and respective discussion and interpretation can be offered.

7. PROSPECTIVES

Preventive or therapeutic vaccines are based on the use of specific antigenic components of *Echinococcus* sp.. The Eg95 vaccine has already reached field validation for its use in *E. granulosus* infection of sheep (Heath et al., 2003), and similar or novel antigens could be adapted to *E. multilocularis* infection of intermediate hosts. In murine AE, preliminary experimental reports suggest that several antigenic compounds may provide good protection against primary infection, for example, Em14-3-3 (Siles-Lucas et al., 2003), Em95 (Gauci et al., 2002), EMY162 (Kouguchi et al., 2007) and EmTetraspanin (Dang et al., 2009). Dependent of the economical and ethical feasibility of such an approach, a preventive vaccine for *E. multilocularis* infection in humans may theoretically be envisaged, while a therapeutic approach still deserves further detailed investigation.

7.1 Prospectives to future immunotherapeutical strategies (what we can learn from 'host resistance to infection')

AE: The notion that Th1-related immune responses could be crucial for efficiently combating *E. multilocularis* metacestode was followed up in mice by experimental administration of cytokines. Treatment with IL-12 was very effective in impairing *E. multilocularis* metacestode development; an interesting observation was the constitution of a fibrous shell around the LL layer in the liver of mice which were found with dead AE lesions; this shell was very similar to the 'adventitia' of CE cysts; unfortunately, IL-12 cannot be used in humans because of its systemic toxicity (Emery et al., 1998). IFN-γ was also tested but revealed only low efficacy (Liance et al., 1998). Temporary stabilization of lesions was observed in human AE patients during IFN-γ treatment. However, prolonged administration was not able to reverse the Th2-biased cytokine profile (Jenne et al., 1998). The most promising immune modulation comes from the observation of one patient with AE and HCV hepatitis who was not treated with albendazole because of adverse effects and received aα. This resulted in a shift in the cytokine profile towards Th1, which was associated with a significant regression of lesions

(Harraga et al., 1999). A marked reduction of larval lesions and modulation of cytokine secretion and effector cell functions were also observed in experimentally infected mice treated with IFN-α-2α (Godot et al., 2003). However, although several efforts on developing immunotherapeutic approaches were undertaken and generated interesting results, they were never followed up by clinical trials. Further improved knowledge about immune modulatory, suppressive or host-protective processes associated with chronic or abortive AE are needed, those include Treg cells (Pan et al., 2013; Wang et al., 2015a), proteins secreted in vivo by *Echinococcus* spp. which inhibit, e.g., protease activities and granulocyte chemotaxis and recruitment (Shepherd et al., 1991), the identification of helminth-derived molecules which induce suppressor cells, either alternatively activated MØs (Jenkins and Allen 2010; Nutman et al., 2015), innate lymphoid cells (Koysu and Moro 2013) which drive and profile antihelminth immune responses (Riner et al., 2013), or distinct subsets of granulocytic MDSCs (Van Ginderachter et al., 2010; Saleem et al., 2012) exerting both immunosuppressive and immunosupportive functions.

CE: As for AE, Th1-related immune responses could be crucial for efficiently combating CE and parasite-derived molecules such as mucins isolated by LL have been studied for their immunomodulatory effects as reported earlier (Noya et al., 2013). However, also for CE, clinical studies based on immunotherapeutic strategy are still lacking and chemotherapy or interventional procedures (e.g., surgical resection, PAIR, etc.) remain the primary choices of treatment in CE. A better understanding of hydatid immunoregulation in CE may pave the way to rational immunotherapy and future vaccine development.

7.2 Combining immunotherapy with chemotherapy?

Without therapy, AE becomes fatal in approximately 94% of patients within 10–20 years following diagnosis (Jura et al., 1998). Radical surgery is the basis of curative treatment for early AE, but patients not suitable for surgery, and those who underwent partial or palliative surgery, require lifelong treatment with benzimidazoles (albendazole, mebendazole) (McManus et al., 2012). Benzimidazoles are predominantly parasitostatic and thus do not kill *Echinococcus* species immediately; however, long-term benzimidazole treatment may become fully effective (Ammann et al., 2015), but respective effector mechanisms (direct or indirect toxicity, or/and immune mediation) are still unknown. Medication of AE with benzimidazole derivatives is a

lifelong endeavor, harboring the risk of deleterious side effects such as liver damage (Kern, 2010). The situation of CE regarding efficient drugs is rather similar, even though benzimidazoles are parasitocidal in vitro: only a percentage of CE cysts respond to benzimidazoles, and in most inoperable patients the treatment must be prolonged for months and even years; this is particularly true for multiple cysts and/or extrahepatic locations of the cysts. Several alternative drugs have been tested in vitro and in vivo, mostly against *E. multilocularis* metacestode so far (Hemphill et al., 2014; Küster et al., 2015), but none has currently been tested in clinical trials.

Large-scale screening for new compounds by means of in vitro maintained cestode larval stages or primary cell culture systems is being applied to identify new molecules potentially effective against proliferating metacestodes, and this may open possibilities to interfere with AE disease progression (Hübner et al., 2010; Hemphill et al., 2014, Brehm and Koziol 2014). Notably the experimental use of drugs, commercially available and FDA-approved, which inhibit cancer cell proliferation and are being applied for cancer treatment, has identified novel and potentially beneficial treatment options, however, their efficacy against *E. multilocularis* has yet to be confirmed in vivo.

Results of observations in humans and experimental studies in animals also suggest that in the absence of fully effective antiparasitic chemotherapy for echinococcosis, immunotherapy, i.e., modulating the host immune response, could be an attractive alternative or complementary treatment option. For this it is required to trace the efficient immune pathways of infection-resistant persons and to compare them to those of immunocompetent susceptible patients, as well as to those of immunosuppressed patients who got thus more susceptible to disease.

Studies carried out on host immunological and genetic factors have progressed to the state where susceptibility versus resistance, disease progression or regression and prognosis after treatment are considered to be influenced by cytokine, chemokines, growth factors and antibody response profiles, and by T regulatory cells (Tregs) which modulate the expression of immune competence. Advanced genomic approaches, such as genome-wide association studies, gene expression studies in patients at distinct stages of echinococcosis may deliver genetic traits; identify functional pathways and response profiles associated with susceptibility, disease progression or regression and progress to new therapeutic interventions (Yang et al., 2012).

Genomic studies that yield candidate target molecules and define the pathways and cell types regulating immune response may transfer to and

find application for echinococcosis, e.g., the immune genetic identification of parasite antigens (peptides) being presented by MHC complexes was successfully developed and is currently applied as tumour-associated antigen therapy for the treatment of several malignancies (Klug et al., 2009), and immunotherapies with immune checkpoint inhibitors and adaptive cell transfer have demonstrated safety and feasibility in hepatocellular carcinoma patients (Tsuchiya et al., 2015), melanoma, Hodgkin disease, renal cell carcinoma and lung and bladder cancers (Shin and Ribas 2015). For example, immunoactive *E. multilocularis* metabolites (e.g., EmTIP) represent interesting targets for immunotherapy of AE and their specific inhibition or neutralization by specific antagonist may inhibit *E. multilocularis* primary cell proliferation. Yet, for AE these immunogenetic approaches and therapeutic strategies await their in vivo application.

The immunological host–parasite relationship has been more extensively studied in the past decade. The main cytokines involved in immune tolerance with regard to AE patients are IL-10 and TGF-ββ. IL-10 is spontaneously hypersecreted in patients with progressing AE. The relevance of TGF-ββ was suspected in patients with AE and has recently been emphasized by several studies in the rodent model. Alternatively, attempts at enhancing Th1-related immune responses using IL-12 or IFN-α-2a resulted in increased resistance to *E. multilocularis* infection in mice. IL-12, however, cannot be used in humans; but IFN-α-2a is a reasonable therapeutic option, especially for patients in whom side effects of albendazole abrogated its use for a continuous treatment of AE; whether IFN-α-2a would also be suitable to treat severe CE should be experimentally tested.

Studies of the cellular response profile in AE patients at distinct states of infection have shown that not only cytokines but also chemokines may orchestrate progression or regression of disease; the Th2-type chemokines TARC/CCL17, LARC/CCL20 and MCP3/CCL7 associated with AE progression, while proinflammatory PARC/CCL18 and MCP4/CCL13 were prominent with AE cure. Such better understanding of the immune response during progression and regression of AE may open preventive or supportive immune therapeutic intervention strategies.

Any immunity-related treatment regimen should be tested in preclinical models. Consequently, more basic studies are necessary to better understand the host–parasite relationship when intermediate hosts of the parasite are treated with immune modulators and to search for new targets. Respective candidates and targets have been elucidated recently, such as Tregs and their effector molecule FGL2 from the patients' perspective or

EmACT and EmTIP from the parasite's perspective. But the final solution will most likely not be found in one or a few single parameters, but rather in a complex and subtle interplay that is orchestrated by parasite metabolites versus the distinct expression of host immunity, and thus in a combination of approaches, such as those currently successfully used to treat cancer. Thus, subtly affecting parasite metabolism with appropriate drugs and simultaneously cleverly channeling the host immune response out of anergy towards a parasitocidal effector pathway might well be the future solution.

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CHAPTER TWO

Echinococcosis: Control and Prevention

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Abstract

Human cystic echinococcosis (CE) has been eliminated or significantly reduced as a public health problem in several previously highly endemic regions. This has been achieved by the long-term application of prevention and control measures primarily targeted to deworming dogs, health education, meat inspection, and effective surveillance in livestock and human populations. Human CE, however, remains a serious neglected zoonotic disease in many resource-poor pastoral regions. The incidence of human alveolar echinococcosis (AE) has increased in continental Europe and is a major public health problem in parts of Eurasia. Better understanding of wildlife ecology for fox and small mammal hosts has enabled targeted anthelmintic baiting of fox populations and development of spatially explicit models to predict population dynamics for key intermediate host species and human AE risk in endemic landscapes. Challenges that remain for echinococcosis control include effective intervention in resource-poor communities, better availability of surveillance tools, optimal application of livestock vaccination, and management and ecology of dog and wildlife host populations.

1. INTRODUCTION

By the mid 19th century the aetiology of human cystic hydatidosis and colloid 'carcinoma' were recognized to be of helminthic parasitic origin caused by a cestode(s) (see Chapter: Immunology of Alveolar and Cystic Echinococcosis (AE and CE) by Gottstein et al., 2017). However, whether both diseases were caused by different forms of *Echinococcus granulosus* or by two separate species was not fully confirmed until the life cycle biology and pathology of *Echinococcus multilocularis* in wild mammals was determined in the 1950s (Rausch and Schiller, 1951; Vogel, 1955). In contrast, the life cycle of *E. granulosus* in domestic mammals was already delineated in 1863 after experimental infection studies by Von Siebold and Naunyn

(see Grove, 1990). Furthermore, the public health importance of CE (hydatidosis) in continental Europe and Iceland in the mid 19th century was significant enough for hydatid disease control recommendations to be published in 1854 and 1863 (Grove, 1990). This was followed by an ultimately successful long-term national health education programme against CE in Iceland (1863–90) (Craig and Larrieu, 2006). The earliest control programme for human alveolar echinococcosis (AE) occurred on Reuben Island in northwest Japan from the 1940s when the fox population was deliberately eliminated to eradicate transmission (Ito et al., 2003a). Currently (2016) both Iceland and Reuben Island (Japan) remain free from echinococcosis and still maintain strict controls on dog registrations and/or movement.

Despite several highly successful hydatid control programmes from the 1960s, primarily in regions with relatively well-developed agricultural sectors (e.g., New Zealand, Tasmania, Cyprus, Chile), human CE remains a significant public health problem in the early 21st century over large pastoral areas in South America, North Africa, Eastern Europe, the Middle East, Central Asia, Russia and China (WHO/OIE, 2001; Alvares Rojas et al., 2014). Total numbers of human cases are >>1 million with an associated significant high disease burden (Budke et al., 2006). The highest burden of human CE occurs over a large more or less contiguous transmission zone from North Africa, Near East, Middle East, Central Asia, eastern Russia and western China (Budke et al., 2006; Craig et al., 2007a). Over this endemic zone, however, only a few CE control programmes are currently active (e.g., western China) or planned (e.g., Tunisia) (WHO, 2010a).

At least 18 echinococcosis control programmes to reduce human CE incidence, have been implemented in different world regions since the 1960s, of which 3 were at national level (New Zealand, Cyprus, Uruguay) and the others at provincial level (Table 1). In all of those programmes the key element or control tool initially or eventually applied was the supervised dosing of owned dogs with a praziquantel(PZQ)-based anthelmintic at a frequency of 4–8 times per year (Gemmell et al., 2001; Craig and Larrieu, 2006; Lembo et al., 2013). Five island-based CE control programmes, including Iceland (from 1863), New Zealand, Tasmania, Cyprus and the Falkland Islands (Las Malvinas), were highly successful in eliminating human CE as a public health problem, with all immediately or eventually applying dog-targeted interventions (including culling, purgation and/or anthelmintic treatments) (Gemmell and Roberts, 1998; Economides et al., 1998; Craig and Larrieu, 2006). By contrast several continental-based CE

Table 1 Summary of control programmes for cystic echinococcosis

Program	Period	Option Auth.	Screen	Pre	~10y	~20y	Final status 2015	References
Iceland	1863–1960	2 (Gov)	Humans	22%		No new	Eliminated	Dungal (1957)
New Zealand	(1939 start) 1959–2002	(2) 5 (hon) (MoA)	Humans[a]	4.5	3.2	0.7/0	Elimination declared 2002	Gemmell (1990) and Craig and Larrieu (2006)
			Sheep (%)	48	0.4–15.6	<0.001		
			Dogs (%)	37	<5	<0.002		
Tasmania	1965–96	3 (MoA)	Humans	>90	28 cases	0	Elimination declared 1996 (reemerging?)	Beard et al. (2001) and Jenkins et al. (2014)
			Sheep	52	8.7	<0.002		
			Dogs	12.7	0.8	0.06		
Cyprus	1971–85	4 (MoA)	Humans	12.9	0	8ˆ	Reemerged (ˆN.Cyprus)	Economides and Christofi (2000) and Christofi et al. (2002)
			Sheep	66	0.9	0.014		
			Dogs	6.8	0.02	2.6		
Falkland Is. (Malvinas)	1965–2010	(3) 5 (MoA)	Humans	55		0	Near elimination	Christofi et al. (2002) and Lembo et al. (2013)
			Sheep	59	1.8	0.16		
			Dogs	?	1.7ˆ	<0.01ˆ (coproag.)		
Sanpete Utah, USA	1971–81	3 (RI) (State)	Humans	3.7		0 new	Near elimination	Loveless et al. (1978) and Andersen et al. (1981)
			Sheep	7.1	2.8	0		
			Dogs	28.3	9.8	0		
Neuquen Argentina	(1970 start) 1995–2004	(3), 5 (MoH)	Humans	22.1		6.2–28	Continued transmission	Larrieu and Zanini (2012) and Larrieu et al. (2004a,b)
			Sheep	76	26.1	2.1		
			Dogs	28	3	1.2		
Rio Negro Argentina	1980–2003	(3),5 (6) (MoH)	Humans	5.6	1.8	0.3	Continued low transmission (ˆ7.8 in <3y old sheep)	Larrieu and Zanini (2012), Larrieu et al. (2000a)
			Sheep	61	2.9	20ˆ		
			Dogs	41.2	18	5.2		

(*Continued*)

Table 1 Summary of control programmes for cystic echinococcosis—cont'd

Program	Period	Option Auth.	Screen	Pre	~10y	~20y	Final status 2015	References
Uruguay	(1965 start) 1994–2002	(3), 5 (hon) (MoH)	Humans	12.4	6.5		Continued transmission	Larrieu and Zanini, 2012 and Cabrera et al. (2002a,b)
			Sheep	41	9	7.6		
			Dogs	10.1	0.7			
Chile XII	1979–97	5 (MoA)	Humans	80	20		Reemergence	Craig and Larrieu (2006), Larrieu and Zanini (2012), and Vidal et al. (1994)
			Sheep	60	25	5–7		
			Dogs	71	5	0.3		
Rio Grande Brazil	1983–2000	5 (MoA)	Humans[b]	1.7			Continued transmission	Larrieu and Zanini (2012) and Farias et al. (2004)
			Sheep	26		3		
			Dogs	28.3	9	25		
Powys Wales	1983–89	5	Humans	3.9	2.3	0 < 15y	Reemergence In dogs	Palmer et al. (1996) and Buishi et al. (2005b)
			Sheep	23.5	10.5			
			Dogs	4–25	0	9		
Turkana Kenya	1983–2000	(4), 5 (NGO)	Humans[b]	9	7	3	Continued transmission (ˆcoproag.)	Macpherson and Wachira (1997), Njeroge et al. (2000) and Buishi et al. 2006
			Goats	2–30	2.5[b]	1.8[b]		
			Dogs	63	27	31ˆ		
La Rioja Spain	1987–2000	(4),5 (MoH)	Humans	19	4	2.46	Continued low transmission	Jimenez et al. (2002) and Carmena et al. (2008)
			Sheep	82.3	20			
			Dogs	7	0.2			
Sardinia	1987–2000	(2), 5 (RI)	Humans	16	9.8		Continued transmission	Craig and Larrieu, 2006 and Conchedda et al. (2002)
			Sheep	85	87			
			Dogs	24	25			
Hutubi China	1987–90	5 (RI) (MoA)	Humans	43.8			Ongoing national prog	Andersen et al. (1991) and Zhang et al. (2009b)
			Sheep	88.8	5.6	4.7		
			Dogs	18.5	0			

Datangma China	2000–2005	5, (6) (NGO)	Humans[b]	1–2.6		Ongoing national prog	Heath et al. (2006)and Yang et al. (2009)
			Yak		38		
			Dogs	50ˆ	17ˆ	(ˆ coproag.)	
Shiqu China	2006-Current	5 (MoH)	Humans[b]	3.3		Ongoing (ˆ coproag.)	Wang et al. (2008) and Moss et al. (2013)
			Sheep	>50			
			Dogs	8 (21ˆ)			

2, main Option applied (see Table 2 for definitions); *(2)*, previous Option used; *Auth.*, Authority responsible; *hon*, Honorary Commission; *MoH*, Ministry of Health; *MoA*, Ministry of Agriculture; *NGO*, Non Government Organization; *RI*, Research Institute.

[a]Incidence – cases per 100,000.

[b]Ultrasound prevalence %

Modified from Craig, P.S., Larrieu, E., 2006. Control of cystic echinococcosis/hydatidosis: 1863–2002. Adv. Parasitol. 61, 443–508; Larrieu, E., Zanini, F., 2012. Critical analysis of cystic echinococcosis control programs and praziquantel use in South America, 1974–2010. Rev. Panam. Salud Publica 31, 81–87.

control programmes ranged from highly successful (e.g., Region XII, Chile; Rio Negro, Argentina; La Rioja, Spain), eventually successful (e.g., Uruguay) to more limited impact (e.g., Turkana, Kenya; mid-Wales, UK) (Craig and Larrieu, 2006) (Table 1).

Modern control of *E. multilocularis* transmission in wildlife cycles is much more difficult to implement compared to *E.granulosus* in its domestic animal cycles because it requires targeted anthelmintics to the fox population. Distribution of baits containing PZQ in endemic areas can have a significant impact on vulpine prevalence, but logistics, cost and sustainability are difficult to maintain over long periods and over large geographic areas (Hegglin and Deplazes, 2013). Where domestic dogs have an important role in zoonotic risk for human AE, frequent deworming of owned dogs should be implemented for public health reasons (Rausch et al., 1990; Wang et al., 2014).

A critical aspect of echinococcosis control has been the appropriate surveillance of both human CE or AE disease incidence or prevalence, livestock prevalence for CE and canine or vulpine echinococcosis prevalence. Without adequate surveillance data at baseline and at quarterly or annual periods post intervention, the impact of control measures will be difficult or impossible to assess and thus justify ongoing expenditure to maintain costly interventions.

Since the 1970s new tools and approaches have become available to assist in planning and implementation of interventions and surveillance strategies. These include an excellent antiworm drug (PZQ) for dogs and foxes (baits); use of portable ultrasound for human screening (CE and/or AE) within endemic communities; a highly effective vaccine (EG95) to prevent ovine echinococcosis; a laboratory-based test [coproantigen enzyme-linked immunosorbent assays (ELISA)] to replace the arecoline purgation test in dogs and to test fox scats; computer-based modelling of cost-benefit for interventions; and transmission dynamics and predictive modelling for intervention combinations (Torgerson and Heath, 2003; Craig et al., 2007b). The reasons for success in some CE control programmes and variable impacts for others are important issues that have been discussed (e.g., Gemmell, 1990; Craig and Larrieu, 2006) but received only little critical analysis (Larrieu and Zanini, 2012; Lightowlers, 2012).

1.1 Basis for prevention and control

The life cycle biology of all taeniid cestodes, including *Echinococcus* spp., has evolved through predator–prey transmission between carnivore and

herbivore mammalian hosts. Though similar in this respect *Echinococcus granulosus senso lato* and *E. multilocularis* differ in utilization of intermediate hosts, i.e., ungulate versus rodent/small mammals respectively. Transmission of *E. granulosus s.l.* is now primarily represented globally by cycles between dogs and domestic livestock, while *E. multilocularis* transmission occurs primarily within wildlife cycles between fox definitive and rodent intermediate hosts. From a control perspective the main target for intervention of both *Echinococcus* species is the definitive host (dogs or foxes) with the aim to reduce or eliminate adult worm burdens. The anticestode drug PZQ provides an excellent cestocidal deworming tool for dogs and foxes but the logistics of regular mass treatment are challenging. Dogs are also an excellent host for *E. multilocularis* and as such may increase zoonotic risk. Targeting intermediate hosts for CE control may be undertaken through classical meat inspection at slaughter but also using an infection preventive vaccine (EG95). There are currently no usable vaccines for definitive hosts. In contrast control measures directed against small mammals would not usually be considered economic or ecologically sound. Treatment of human CE and AE cases may be a public health priority but will not directly affect transmission because humans are almost always 'dead-end' hosts. Health education has the potential to reduce risky behaviour of humans, for example, unhygienic slaughter and dog contact, but education is also probably more important for community acceptance and voluntary participation in long-term hydatid-control programmes.

2. TARGETS, OPTIONS AND TOOLS FOR CONTROL OF *ECHINOCOCCUS GRANULOSUS*

At any one time transmission of *E. granulosus* is dependent on presence of viable parasite stages in dogs or other canids (adult tapeworms), domestic ungulates or wild herbivores (metacestodes) and the environment (eggs). As stated, human infections with the metacestode (CE) do not normally contribute to active transmission (because it requires a dog to ingest hydatid cysts). Removal or reduction in worm biomass in definitive hosts (usually dogs) will have the greatest and quickest effect to reduce active transmission because egg production will decrease rapidly and thus infection pressure to livestock. This will also importantly reduce the direct zoonotic risk from dogs within endemic communities. Targeting livestock to prevent infection (anti-oncosphere vaccination) or to kill hydatid cysts

(anthelmintics) could also be effective especially in conjunction with slaughter inspection (liver/lungs condemnation) and husbandry practices that reduce numbers of older sheep (have the greatest viable metacestode burden). Simulation models for combined deworming of dogs and vaccination of sheep indicate improved efficacy (Torgerson and Heath, 2003; Lightowlers, 2012). Community knowledge about the life cycle of *Echinococcus* and risks of infection from dogs should also be beneficial in relation to preventative behavioural changes (e.g., not to feed dogs raw offal). Where hydatid control measures can be integrated with the control of other zoonotic diseases or public health programmes (i.e., 'One Health'' approaches), this is expected to be more efficient and cost-effective (Narrod et al., 2012), although so far there are few examples of this regarding CE (Zinsstag et al., 2006; Rabinowitz et al., 2013).

2.1 Control approaches, options and phases for cystic echinococcosis control

The control measures formulated in the 1860s (Krabbe, 1864) to reduce the transmission of *E. granulosus* between dogs and sheep are still valid today. The four key components were:

- prevent dogs getting access to offal,
- treat dogs with a dewormer,
- meat inspection and offal disposal, no home slaughter,
- health education about hygiene and dog contact.

These control directives were applied nationally in Iceland from 1863, and were supported in the early 20th century by a change in sheep husbandry in Iceland towards marketing fat lambs rather than sheep production for milk and cheese, so that human CE cases significantly reduced within 30 years and transmission was eliminated from Iceland after ~100 years (Dungal, 1957; Grove, 1990; Craig and Larrieu, 2006). Between the 1950s and 1970s another four island hydatid control programmes were initiated (i.e., New Zealand, Tasmania, Falkland Islands, Cyprus) and were all ultimately highly successful (Gemmell, 1978; Gemmell and Roberts, 1998). They all targeted dogs in vertical programmes to varying degrees, but with some differences. For example, annual arecoline testing of dogs followed by euthanasia (Cyprus) or by enforced quarantine (Tasmania) of positive animals, or the supervised six weekly dosing of dogs with PZQ (New Zealand, Falkland Islands). From the 1980s the use of regular PZQ dosing of dogs was also the key intervention tool in several continental hydatid control programmes in

South America (Argentina, Uruguay, Chile, Brazil), in Europe (Wales, Spain), in East Africa (Kenya), and in Asia (China, Kyrgyzstan).

From these programmes, but especially the Island-based schemes, a list of five 'control options' for CE was considered by Gemmell (1978), then formulated by Gemmell and Lawson (1986) and further extended to include potential livestock vaccination, i.e., an 'Option 6' (Craig and Larrieu, 2006) (Table 2). Option 5 that is based on the regular dosing of owned dogs with PZQ, has been the preferred option, since the drug became readily available in the 1980s to enable a 'fast-track' approach (Gemmell and Roberts, 1998). The challenge for control authorities was and is the existing infrastructure capability, available veterinary-related manpower, effective outreach to access all owned dogs (4–8 times per year) in target rural communities in often remote resource-poor areas, and that the communities are furthermore compliant with the control scheme. The lack of obvious clinical signs of infection with *E. granulosus* in both livestock and dogs, coupled with the relatively low direct economic impact in livestock, means that there is often little priority especially for poor livestock keepers within endemic communities, and also low priority for animal health sectors biased towards more economic problems in livestock (Craig et al., 2007a).

In common with some other neglected zoonotic diseases (NZDs) the complication with CE is that it is foremost a public health problem rather than an animal health problem. Thus consideration for control is a human health priority and although a ministry of health can collect hospital data (i.e., surgical cases per 100,000 year), it relies on cooperation with the veterinary sector (e.g., Ministry of Agriculture) to deliver control measures and also to undertake domestic animal surveillance (WHO, 2010a,b). Intersectoral cooperation that is beneficial is frequently difficult to initiate and sustain (Gemmell, 1978; Marcotty et al., 2013). Hydatid control schemes/programmes have been delivered by a ministry of agriculture (e.g., Tasmania; Region XII, Chile), a department of veterinary services (e.g., Cyprus), a ministry of health (e.g., Rio Negro, Argentina; western China), an honorary commission (e.g., New Zealand; Uruguay), or a nongovernment organization (e.g., Turkana, Kenya). In all cases some degree of cooperation between the veterinary and medical sectors was required to make a decision to initiate planning for hydatid control and to then collect animal and public health surveillance data respectively. Table 3 lists the phases of control that could be considered: planning phase, attack phase, consolidation phase and maintenance of elimination (Gemmell et al., 1986b, 2001; Gemmell and Roberts, 1998).

Table 2 Options considered for control of cystic echinococcosis

Option	Main components	Period required	Examples	References
1	Decision not to proceed	Planning only		Gemmell and Roberts (1998)
2	Horizontal measures, health education, upgrade of abattoirs, changes in husbandry	>50 years	Iceland (from 1863) New Zealand (before 1959)	Dungal (1957) Gemmell (1990)
3	Arecoline testing, quarantine, health education	10–20 years	Tasmania (1965–96) Utah (1971–81) Uruguay (before 1994)	Beard et al. (2001) Andersen et al. (1981) Gemmell et al. (2001)
4	Dog population control and euthanasia arecoline testing	10 years	Cyprus (1971–85)	Gemmell et al. (2001)
5	PZQ dosing dogs (4–8 x p.a), meat inspection, health education	<10 –>20 years	New Zealand (from 1960) Chile XII (1979–97) Rio Negro (1980–2003) La Rioja (1987–2000) Mid-Wales (1983–89)	Economides et al. (1998) Gemmell and Schantz (1997) Larrieu et al. (2000a) Jimenez et al. (2002) Palmer et al. (1996)
6	PZQ dosing dogs (2 x p.a), livestock vaccine, health education	? <10 years	Datangma (2000–05) Rio Negro (2010–15)	Heath et al. (2006) Larrieu et al. (2015)

PZQ, praziquantel; *p.a.*, per annum.
After Gemmell, M.A., Roberts, M.G., Beard, T.C., Campano Diaz, S., Lawson, J.R., Nonnemaker, J.M., 2001. Chapter 6: control of echinococcosis, In: Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (Eds.), WHO/OIE Manual on Echinococcosis in Humans and Animals : A Public Health Problem of Global Concern. WHO/OIE, Paris, France; Craig, P.S., Larrieu, E., 2006. Control of cystic echinococcosis/hydatidosis: 1863–2002. Adv. Parasitol. 61, 443–508.

Table 3 Phases considered for cystic echinococcosis control programmes

Phase	Period/start	Key elements
Planning	1−5 years before start	Decide on Option (1−6) and approach, cost-benefit analysis, burden of human disease, funding sources and expectation for 5−10 years, identify control authority, integrated measures, select intervention region and communities, applied research needs, participatory planning, outreach ability, transport, quality of baseline data (humans, livestock, dogs), surveillance options, registration of households and dogs, stray dog issues, select staff required, training, health education aspects, medical support treatment and follow-up of CE cases, intersector cooperation. May also consider a pilot scheme.
Attack	1−5 years, 5−10 years, >10 years	Intervention/control measures applied, specified dosing frequency (PZQ, arecoline) minimum 2−4 p.a for PZQ, dog population control, setting-specific health education, slaughter inspection and condemnation, use of livestock vaccine, husbandry aspects, age-specific surveillance data for humans and livestock, arecoline or coproantigen testing of dogs.
Consolidation	Year 8−10, or >10 years after start	Attack phase ceased, transfer to surveillance with infected livestock trace-back, application of quarantine on positive properties, possible reintroduction of dog-dosing measures, provision or voluntary purchase of PZQ for dog owners, use of sentinel livestock, possible legislation against home−slaughter and animal movement, incorporation of EG95 into routine vaccine schedules. This phase may need to be permanent.

(Continued)

Table 3 Phases considered for cystic echinococcosis control programmes—cont'd

Phase	Period/start	Key elements
Maintenance	20 to >30 years after start	A permanent phase applied when control activities stopped and when elimination is close or been declared, maintain vigilance by meat inspection (identify small lesions) and hospital records (for children), trace-back when required, movement controls (licence or passport) on dogs especially for islands. Definitions used for elimination.

See reviews Gemmell, M.A., Schantz, P.M., 1997. Formulating policies for control of *Echinococcus granulosus*: an overview of planning, implementation and evaluation. In: Andersen, F.L., Ouhelli, H., Kachani, M. (Eds.), Compendium on *Cystic Echinococcosis* in Africa and in Middle Eastern Countries with Special Reference to Morocco. Brigham Young University Print Services, Provo, Utah, USA. pp. 329−345; Gemmell, M.A., Roberts, M.G., Beard, T.C., Campano Diaz, S., Lawson, J.R., Nonnemaker, J.M., 2001. Chapter 6: control of echinococcosis, In: Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (Eds.), WHO/OIE Manual on Echinococcosis in Humans and Animals : A Public Health Problem of Global Concern. WHO/OIE, Paris, France; Lembo, T., Craig, P.S., Miles, M.A., Hampson, K.R., Meslin, F.X., 2013. Zoonoses prevention, control, and elimination in dogs, In: Macpherson, C.N.L., Meslin, F.X., Wandeler, A.I. (Eds.), Dogs, Zoonoses and Public Health. CABI, Wallingford, UK, pp. 205−258.

2.1.1 Definitions of control

To establish whether a reduction in pathogen transmission has been achieved as a result of a purposeful control campaign, the concepts of 'control', 'elimination' and 'eradication' require consideration (Gemmell, 1986; Molyneux, 2006).

For echinococcosis, the elimination of human CE disease is the ultimate goal, i.e., no human cases in a defined geographic region. However, because CE patients may have a long asymptomatic period, cases will continue to appear in older age groups (e.g., >40-years-old) long after elimination has been declared. This was the situation in Iceland, New Zealand and Tasmania, wherein CE cases in older age groups continued to occur some years after elimination was considered to have occurred (Craig and Larrieu, 2006; Moro and Schantz, 2006a,b; O'Hern and Cooley, 2013). Elimination of infection or transmission, as opposed to disease in humans, is more difficult because it requires that there is no infection in domestic animals (dogs and livestock) and possibly wildlife hosts, however, that may be the ultimate aim of an echinococcosis control programme. For example, provisional elimination of *E. granulosus* transmission was declared in Tasmania in 1996, a total of 32 years after the start of a hydatid control programme

(Jenkins, 2005), and in 2002 in New Zealand 43 years after implementation of a vertical control programme (Pharo, 2002). However, in Tasmania infection in cattle and dogs was detected after 2006, suggesting some transmission of *E. granulosus* between dogs and cattle and/or possibly also involving hunting dogs and wild herbivores (Jenkins et al., 2014). No new human CE cases have been reported in Iceland since the 1960s nor on the Falkland Islands (Malvinas) since 1992 after the start of control programmes respectively in 1863 and 1977 (Lembo et al., 2013).

Pathogen eradication has been defined as the worldwide reduction to zero cases, for example, as in the case of smallpox or the potential near-future eradication of human dracunculiasis (Molyneux, 2006). For zoonotic diseases, such as echinococcosis, with animal host reservoirs this would be virtually impossible to achieve unless within restricted geographic areas such as islands. For most echinococcosis control programmes in continental regions, successful 'control' will usually be considered to be a significant reduction in the incidence, prevalence and morbidity of human CE to some low level. In other words, to eliminate CE as a public health problem, or effectively converting a region of high CE endemic status to one of sporadic CE disease status, and possibly eventual elimination altogether of human disease was achieved in New Zealand and the Falkland Islands.

Following an 'attack' phase characterized, for example, by 5–10 years of regular dosing of owned dogs, and improved husbandry and slaughter practices, as well as health education, the conversion of a hydatid programme to a 'consolidation' phase characterized by surveillance and trace back, can occur when an acceptable level of transmission reduction has occurred (Heath et al., 2006). This could be when there are no new human CE cases under 10–15 years of age (hospital records or ultrasound screening data), when ovine CE prevalence is <0.1% in sheep less than 3-year-old (abattoir records) and when prevalence of canine echinococcosis is < 0.01% (arecoline or coproantigen data) (WHO, 2011).

2.2 Targetting dogs for control of *Echinococcus granulosus*

Since the domestic dog was identified as the main definitive host of *E. granulosus* by the early 1860s, it became clear that reduction/elimination of dog populations and/or more acceptably treating dogs with a dewormer or anthelmintic, could break the life cycle of the parasite and reduce human exposure (Gemmell, 1990; Grove, 1990). The three main approaches involving targeting dogs have been: (1) use of arecoline salts to purge dogs; (2) use of PZQ as an anthelmintic; (3) dog population management.

2.2.1 Arecoline purgation

In 1890 Iceland passed a nationwide law to control dogs by taxation and to enforce treatment with a dewormer. Initially an extract of kamala fruit (*Mallotus philippinensis*) was used as a dewormer but was quickly replaced by a more effective purgative based on extracts of areca nut (*Areca catechu*). A synthetic derivative arecoline hydrobromide (from 1924) was used in dogs at 2–5 mg/kg as a single dose (or with a second follow-up dose), to paralyze tapeworms (*Echinococcus, Taenia* spp and other cestode genera) and to dislodge them through involuntary intestinal smooth muscle contractions by acting on the host parasympathetic nervous system. This resulted in purgation of contents of the intestinal tract including any helminth parasites (Gemmell, 1973; Craig, 1997; WHO/OIE, 2001). Arecoline is not helminthicidal and therefore purged worms are still alive, as will be any tapeworms that still remain attached to the small intestine. Thus a purged dog could still have a small biomass of viable *Echinococcus* worms. However, the potential diagnostic application of arecoline purgation (arecoline testing) was clear and in that regard it has been used successfully as a surveillance tool in several hydatid control programmes from the 1960s and 1990s (see Section 3.2).

Arecoline purgation was employed as both a crude dewormer and a diagnostic test in a number of early hydatid control programmes including: Iceland (from 1890), New Zealand (from 1938), Tasmania (from 1964), Uruguay (from 1965), Neuquen (from 1970) and Cyprus (from 1971) (Gemmell, 1978; Craig and Larrieu, 2006). The two great advantages of arecoline purgation were firstly its 'on-site' educational value to enable dog owners to observe whether their dogs were infected with tapeworms (especially the common large *Taenia* spp), and secondly that purgation should be 100% specific for *E. granulosus s.l.* (i.e., where *E. multilocularis* is not coendemic). However, the logistics of mass arecoline purgation of owned dog populations is very demanding and requires good organization, rural population compliance, timed notice of treatment schedules, trained manpower, purge inspection/analysis ability and biohazard containment in the field (Cabrera et al., 2002b). The sensitivity of single dose arecoline for eliminating *E. granulosus* from infected dogs ranges from 40 to 75% (usually within 30–120 min in dogs starved for 12 h) and therefore a number of dogs will not purge properly or fail to purge. In addition, owner compliance may not be guaranteed because of distressed animals. Since the late 1970s the anthelmintic drug PZQ has been the drug of choice to deworm dogs in *Echinococcus* control programmes (see later). However,

arecoline purgation remains a useful surveillance test for canine echinococcosis when other diagnostic tests are not available and when epidemiological data are required that include mean worm burdens (Ziadinov et al., 2008; Craig et al., 2015).

2.2.2 Praziquantel dosing

The discovery of PZQ in 1972 (by the companies Merck and Bayer) was probably the most important advance for control of echinococcosis since the determination of the life cycle of *E. granulosus* more than 100 years earlier. PZQ (isoquinolone-pyrazine) was found to be highly effective against trematodes and cestodes, including *Echinococcus* and *Taenia* spp.at a dose range of 2–5 mg/kg (Gemmell and Johnstone, 1981). The first use of PZQ for mass treatment of canine echinococcosis began in the late 1970s as six to eight weekly dosing campaigns in the New Zealand Hydatid Control Programme and for hydatid disease control in the Falkland Islands and from the early 1980s in southern Chile (Region XII) and Argentina (Rio Negro) (Craig and Larrieu, 2006). Other successful applications of PZQ dosing of dogs for control of hydatidosis were reported in northern Spain (La Rioja) from 1987 (dosing frequency 6 weekly then 16 weekly from 1993) (Jimenez et al., 2002) and in Uruguay from 1992 again with a target of 6 weekly dosing frequency (Cabrera et al., 1996). From 2006 PZQ manufactured in China was used for an ambitious monthly dosing programme for canine echinococcosis to control transmission in northwest Sichuan Province, then control was expanded in 2010 to include six other provincial regions of western China (WHO, 2011). Application of regular supervised PZQ dosing for hydatid control in remote-settled communities (Larrieu and Zanini, 2012) and in nomadic or semi-nomadic pastoral communities is probably the most challenging (Macpherson and Wachira, 1997; Heath et al., 2006; Huang et al., 2008; Lembo et al., 2013).

PZQ is normally given to dogs orally in tablet form at a dose of 5 mg/kg, though lower doses have been used, as have biscuit (Chi, 1993) and injectable formulations. Its efficacy is >99% against *E. granulosus s.l.* and also for *E. multilocularis* but has no residual effect and is not ovicidal. Treated dogs should ideally be restrained before and after dosing, and as a precaution against environmental contamination any voided faecal matter must be buried or burnt.

The selected frequency for dosing dogs with PZQ is an important aspect of echinococcosis control programmes from perspectives of transmission potential, natural reinfection rates and the logistics, sustainability and cost

of application. The prepatent period of *E. granulosus* sensu stricto in dogs (i.e., time from ingestion of protoscoleces in hydatid cysts to egg production by adult worms) is between 42 and 45 days. Thus a six weekly (42 days) dosing frequency would be expected to prevent egg output if all dogs were successfully treated. In reality maintaining a supervised six weekly PZQ dosing programme for >90% of owned dogs in a given region is extremely demanding and costly and especially for resource-poor remote rural areas. Furthermore, dosing all dogs eight times per year may not always be feasible or affordable over the lengthy time periods (5–10 years) required to have a significant impact on transmission. This aspect is one reason why successful long-term hydatid control programmes have tended to be initiated in the richer and better developed agricultural sectors, for example, as occurred in New Zealand, Spain, Argentina and Chile.

Reduction of a PZQ dosing frequency to less than eight times per year has been considered and applied, for example, in the consolidation phase of hydatid control in Chile where prevalence of echinococcosis in dogs and sheep had dropped significantly (Vidal et al., 1994; Gemmell and Schantz, 1997). A lower frequency of dosing may also be selected for other nonscientific reasons, e.g., because of lack of veterinary technicians, difficult outreach, seasonal restrictions, lack of funding and/or change of public health priorities (Craig and Larrieu, 2006). In the western China Echinococcosis Control Programme (from 2006–7) the intended targeted dog-dosing frequency was monthly, however, this is very difficult to achieve especially in scattered semi-nomadic remote communities (e.g., Tibetans, Mongolians, Kazakhs). An independent evaluation of canine echinococcosis was undertaken in northwest China in an area of a control zone that covered Hobukesar Mongol Autonomous County in northwest Xinjiang, and was subject to monthly dosing (van Kesteren et al., 2015). These authors, however, found that 36.8% of dog owners had never dosed their dogs and only 22% of dogs were reportedly dosed within the six week period prior to dog testing by coproantigen ELISA.

Optimization of a PZQ-based dosing programme for canine echinococcosis could also be informed by exposure data about the natural reinfection rate of owned dogs in the target intervention zone (Larrieu and Zanini, 2012; Lembo et al., 2013). Such data, however, are difficult to collect and ideally should be determined in the planning phase of hydatid control (Gemmell et al., 2001). Nevertheless dog reinfection data have been examined for cohorts of owned dogs that were followed up in several endemic regions where hydatid control was already in place, for example,

in Rio Negro, Argentina (Larrieu et al., 2000a) and Shiqu county, China (Moss et al., 2013). Reinfection studies were also undertaken prior to a planned change of intervention in Uruguay from arecoline purgation to emphasis on PZQ dosing (Cabrera et al., 1996). In those studies dog cohorts (>300 dogs per cohort, prevalences at baseline 13.2–42%) were tested using arecoline purge or coproantigen ELISA then treated once with PZQ and followed up at 2, 3–4 and 8–9 months posttreatment (Lembo et al., 2013). Canine echinococcosis prevalences had returned to an equivalent of 50–100% of pretreatment levels by 8–9 months later, and by 11–41% as a proportion of baseline levels after 3–4 months posttreatment when actual prevalences ranged from 2.2% to 6.7% (Table 4). These three different natural reinfection studies suggest that a minimum dog-dosing frequency for PZQ should be every three months, i.e., four times per year, which has also been recommended by WHO (WHO/OIE, 2001; WHO, 2011).

Computer-based models of *E. granulosus* transmission dynamics, though theoretical, can be informative for consideration of minimum dog-dosing frequencies, especially when multiple interventions are applied, for example, the inclusion of the EG95 vaccine for ovine echinococcosis (Torgerson and Heath, 2003; Huang et al., 2011).

2.2.3 Dog population management

The domestic dog is the most important definitive host for *E. granulosus s.s.* and dog ownership and contact are key risk factors for human CE in rural endemic areas (Otero-Abad and Torgerson, 2013; Wang et al., 2014; Craig et al., 2015). Presence of free-roaming owned, community-owned and/or stray dogs in urban or periurban areas may also be a risk factor for human

Table 4 Reinfection studies in dogs from natural exposure to *E.granulosus* after a single PZQ dose

Region	N dogs	Prev. % Day 0	2 months	3–4 months	8–9 months	References
Rio Negro (Argentina)	476	42[a]	3.5	6.7	21.3	Larrieu et al. (2000b)
Florida (Uruguay)	303	13.2[a]	0	5.4	18.6	Cabrera et al. (1996)
Shiqu (China)	329	19.5[b]	9.3	2.2	14.1	Moss et al. (2013)

[a]Arecoline test.
[b]coproELISA.

CE. For example, as was observed for community-owned dogs in central Kathmandu, Nepal (Baronet et al., 1994), or for abattoir dogs in Lima, Peru (Reyes et al., 2012). Therefore managing dog populations to reduce their numbers could in theory help to reduce transmission, especially in conjunction with other measures such as dosing dogs and stricter livestock slaughter practices. Culling dog numbers was recommended in Iceland by Krabbe in the 1860s but not generally accepted, rather in Iceland a law was passed in 1890 to keep dogs outside city boundaries and to treat them with areca extract (Grove, 1990). By contrast, in Cyprus in the early 1970s arecoline purge positive dogs and free-roaming dogs were euthanized by the government veterinary services as part of a hydatid control programme that was highly effective (Economides et al., 1998). Unwanted dogs were also culled as part of a control scheme in Hutubi county northwest China (Zhang et al., 2009b). In the La Rioja hydatid control programme in Spain, stray and uncontrolled dogs were impounded then euthanized, and a sample of animals necropsied annually to provide surveillance prevalence data on canine echinococcosis (Jimenez et al., 2002).

There are, however, significant problems of ethics and logistics as well as availability of humane means to reduce numbers of unwanted dogs (Kachani and Heath, 2014). One feature of most rural endemic areas is that local veterinary, agricultural or municipal authorities do not know the size of owned or stray domestic dog populations because they are not livestock in the economic sense and thus of low priority (Craig et al., 2007a). In the planning phase prior to implementing hydatid control measures, the owned dog population needs to be enumerated and animals registered by household or family group. In addition the size and location of stray dog populations should be determined. One difficulty is that so-called 'stray' populations may include unowned (free-roaming) dogs, but also free-roaming dogs that are family-owned or community-owned (Baronet et al., 1994; Kachani and Heath, 2014; Wang et al., 2014). Failure to adequately include stray dogs in an echinococcosis control campaign may cause problems of intervention efficacy and even premature termination or failure (Conchedda et al., 2002; Jimenez et al., 2002). The average number of owned dogs per household varies in pastoral regions as does the average owned dog turnover rate. In pastoral communities of northwest Sichuan Province and Xinjiang (China), the mean number of dogs per family was 0.86–1.34 (Wang et al., 2006a; Zhang et al., 2009b); 1.8 per household in Morocco (Ouhelli et al., 1997) and 2.2 dogs per household in Limari Province, Chile (Acosta-Jammet et al., 2014).

Attempts to reduce dog populations by culling may not always have a significant impact on dog population densities (WHO, 2011) especially where a canid vaccine is available for other diseases, e.g., rabies and distemper viruses (Lembo et al., 2013). There is no vaccine for canine echinococcosis reinforcing a view that culling dog populations has a role in hydatid control. If dog culling is to be considered in relation to hydatid control there should be general community approval and participatory planning, also cooperation between veterinary and municipality sectors. The latter sector often already has responsibility for culling stray dogs and may respond after public concern, for example, in relation to dog-bites. Culling measures that may be acceptable include barbiturate/anaesthetic overdose, gassing, free bullet or captive-bolt (Kachani and Heath, 2014). Where possible and affordable nonlethal measures should also be considered and these include fertility control by spaying or castration (Macpherson and Wachira, 1997; Economides et al., 2002; Larrieu and Zanini, 2012) or immunocontraception (van Johanssen and Penrith, 2009; Lembo et al., 2013). As mentioned, impounding stray dogs may have public appeal but often is not feasible or affordable especially in resource-poor areas. Dosing stray dog populations with PZQ directly (costly) or in baits (sustainable?) may be factored into a control programme, but will be more difficult and expensive to include. For example, in Tibetan communities in Shiqu county (Sichuan, China) 2500 owned dogs were registered versus an estimated 4400 nonregistered unowned 'community' dogs (Budke et al., 2005b).

2.2.4 Vaccination of definitive hosts of Echinococcus granulosus

Pet and shepherd dogs play a major role in transmission of *E. granulosus* to humans. Dogs are few in number compared to the number of intermediate hosts, and they are often relatively easy to secure to have them vaccinated.

Ample evidence exists for the existence of protective immune responses against hymenolepid cestodes (reviewed by Ito and Smyth, 1987). Adult *E. granulosus* are certainly immunogenic in their hosts (Jenkins and Rickard, 1986), but there is little evidence to support the existence of immunologically mediated protective immune responses against the adult tapeworm. What evidence does exist that indicates the degree of immunity acquired following an initial infection is inconsistent and incomplete, if it exists at all (reviewed by Lightowlers, 1990). In a series of trials undertaken by Gemmell et al. (1986a), 16 dogs were given 8 or 9 repeated rounds of *E. granulosus* infection and treatment, and the number, size and fecundity of the worms developing after each infection were assessed. The results were

very variable both among dogs as well as for individual dogs. Five of the animals appeared to show no diminution in the number or development of tapeworms over the course of the repeated infections. None of the dogs developed a clear level of immunity to the establishment of infection, however, some animals showed a decline in the size and fecundity of worms developing as the number of repeated infections increased. Gemmell et al. interpreted their data to predict that most animals would become resistant by the 12th challenge infection, although this has never been actually demonstrated. One remarkable aspect of the work published by Gemmell et al. is that, while Michael Gemmell had extensive experience working with *E. granulosus* in dogs, possibly more experience than any other person before or since, the variability of the 'takes' of infection between different animals given the same batch of protoscolesces was extraordinarily high. This highlights one of the particular difficulties working with experimental infections in dogs with *E. granulosus* and emphasizes a special need for caution when interpreting the results of challenge infections in dogs because of the high level of variability in the course of infections even in naive animals.

Attempts to induce protection against *E. granulosus* by vaccination with nonliving antigen preparations have seen inconsistent results. Turner et al. (1936) were able to induce partial resistance to *E. granulosus* infection in dogs following immunizations with antigens derived from hydatid cysts. Gemmell (1962) found that worms developing in dogs vaccinated with freeze-dried preparations of either adult tapeworm tissue or scolices were almost always less developed than were the worms in unvaccinated dogs. Herd et al. (1975) described the results of a trial involving two groups of dogs; one group of six animals was vaccinated with antigens secreted in in vitro culture by 33–39 day old *E. granulosus* tapeworms that were maintained in culture for 6–10 days. Five control animals received immunizations with adjuvant alone. The number of worms establishing following a challenge infection was not significantly affected, however, there was a decrease in the proportion of mature worms containing eggs in the vaccinated animals compared with the controls. Vaccination appeared to have caused an arrested, or delayed, development of the worms. A follow-up experiment involving two groups of 10 dogs was unable to repeat the same vaccine-associated effect, with several of the control animals showing what appeared to be the same type of arrested/delayed development (Herd, 1977). These data serve to further emphasize the need for caution in interpretation of data from *E. granulosus* challenge trials

undertaken in dogs. Gemmell et al. (1986a) also observed retarded growth of the whole worm population in some previously naive dogs following an experimental infection with *E. granulosus*.

Zhang et al. (2006) described an antifecundity effect in dogs vaccinated with recombinant antigens. In two experiments, they observed a very high level of protection (97–100%) in terms of suppression of worm growth and, especially, of egg development and embryogenesis. The potential significance of their finding as a potential new tool for control of CE was subsequently highlighted in a review publication (Zhang and McManus, 2008).

E. granulosus worms develop in a relatively synchronous manner following an experimental infection and after shedding a gravid proglottid, appear immature while a new gravid proglottid develops over a period of about 10 days (Heath and Lawrence, 1991). Studies that have described an antifecundity effect in vaccinated dogs have not considered that the effect could potentially have been the result of precocious development stimulated in the vaccinated animals, rather than retarded development. None of the investigations discussed above that described antifecundity effects included data on the examination the dogs' faeces prior to necropsy. Had this been undertaken, it would have indicated or excluded precocious development of worms. There is also no information to indicate whether worms that appeared to be developing relatively slow would progress over subsequent days into fully gravid worms. This information would be vital to the interpretation of any antifecundity effect of vaccination as having a potential use in controlling *E. granulosus* transmission, especially since most studies have failed to detect any effect of vaccination on the numbers of worms establishing.

Petavy et al. (2008) described the use of *Salmonella* vaccine vectors expressing two different proteins of *E. granulosus* and the use of these live, attenuated organisms to immunize dogs against a subsequent challenge infection with the parasite. The paper described a reduced number of *E. granulosus* worms developing in the vaccinated animals. Due to the small sample size used by Petavy et al., there is some controversy as to whether the modest effects that were claimed were statistically significant (Torgerson, 2008). There have been no follow-up publications on this vaccine since the initial publication.

The most impressive results that have been obtained in vaccination against *E. granulosus* infection in dogs were those described by Zhang et al. (2006) in which vaccinated dogs were found to have almost no

mature worms at all, while control animals were all infected with large numbers of mature worms. It is now a decade since these data were presented and no follow-up data have been fully published. Conference proceedings presented by Dr Zhang et al. have indicated that they have been unable to replicate the statistically significant effects previously attributed to the recombinant antigens and, indeed, saw the same comprehensive antifecundity effect in a control group of dogs vaccinated with adjuvant alone (Zhang et al., 2009a).

In conclusion, there appears to be little cause for optimism for the development of an effective vaccine for dogs, with most studies beset by the occurrence of unreliable, nonspecific and unrepeatable effects.

2.3 Targetting livestock for cystic echinococcosis control

2.3.1 Slaughter inspection

Ideally, all agricultural animals that are potential intermediate hosts for *E. granulosus* should be inspected at slaughter. Organs containing hydatid cysts should be removed, condemned and destroyed in a manner that prevents consumption by dogs. Preferably it should be a legal requirement that all livestock should be slaughtered in an abattoir under veterinary control.

A major problem in highly endemic areas of echinococcosis is the clandestine slaughter of livestock resulting in offal being available for consumption by dogs. In many low-income countries where there is livestock pastoralism, organized slaughter facilities may be inadequate or nonexistent with little or no veterinary supervision of slaughter. This combined with lack of knowledge by livestock owners promotes conditions for transmission to dogs. This is well-illustrated in countries of the former Soviet Union where organized slaughter facilities were abandoned when livestock farms were privatized. This resulted in a substantial epidemic of CE across central Asia, the Caucasus and parts of eastern Europe (Torgerson, 2013).

In many upper income countries the incidence of cestode zoonoses has declined with the development of hygienic supervised slaughter facilities. For example, EU Regulation (EC) No 853/2004 (EUR-Lex - f84002 - EN - EUR-Lex, n.d.) laying down specific hygiene rules for food of animal origin states that in most cases meat for human consumption must be from animals slaughtered in an approved slaughterhouse where there is veterinary supervised postmortem inspection. However, such strict regulations are no guarantee that transmission of echinococcosis will be prevented. Italy, for example, has one of the highest incidences of human CE in the European

Union with over 1000 cases treated surgically each year (Brundu et al., 2014). This indicates that there must be infected offal that remains available to dogs despite these regulations being in place. In contrast in Tunisia 80% of butcher's shops slaughter animals and sell the resultant meat and only 13% of butchers had any understanding of the transmission of echinococcosis. Dogs were seen close to 52% of these butcher's shops (Besbes et al., 2003). This well illustrates that illegal slaughtering has an important impact on the transmission of CE. Access to slaughtered animals by dogs is demonstrated by a study from Uganda. Dogs with access to slaughter facilities had a higher prevalence of *E. granulosus* infection. However, if meat inspection was practised at the slaughter facility there was a lower prevalence of infection in the local dog population compared to where there was no meat inspection (Oba et al., 2016).

Also of importance is natural mortality of livestock and the resulting carcasses being available for consumption by dogs. For example, in some areas of the Tibetan plateau there may be a 20% mortality of yaks in the spring. Following recovery of the skin, the dead yaks are discarded and subsequently scavenged by dogs and other carnivores (Hu et al., 2013). This is likely to be a major contributing factor to the transmission of CE in Tibetan communities.

2.3.2 Vaccination of livestock intermediate hosts

Theoretically at least, it would be possible to consider vaccination of humans living in endemic areas for the prevention of CE. However, there is relatively little transmission of *E. granulosus* in the developed world, with the most highly endemic areas being in developing countries. For this reason there is a lack of commercial incentive for the development of a human vaccine and the investment of many hundreds of million dollars that are required to produce, trial and license a new human vaccine. Vaccination of animals to break the parasite's life cycle offers a more practical alternative. Domestic dogs and livestock animals both offer potentially valuable targets for vaccination against *E. granulosus*.

In contrast to the definitive hosts of taeniid cestode parasites, there is ample evidence about the existence of immunologically mediated immunity against infection in the parasites' intermediate hosts, including for *E. granulosus* (Rickard and Williams, 1982). Protective antigens have been identified, produced using recombinant techniques and developed into highly effective vaccines for several species of *Taenia* and for *E. granulosus* (Lightowlers, 2006).

Sheep infected with *E. granulosus* show a resistance to reinfection and there is cross-immunity between the different cestode species infecting sheep (Gemmell, 1966; Heath et al., 1979). Heath et al. (1981) discovered that sheep could be protected against an experimental challenge infection with *E. granulosus* by previous injection of *E. granulosus* oncospheres, or by vaccination with the products of oncospheres secreted in in vitro culture (Osborn and Heath, 1982). The protective oncosphere components were identified (Heath and Lawrence, 1996) and one protective antigen, designated EG95, was cloned from oncosphere mRNA by Lightowlers et al. (1996). The vaccine has proven to be reliable and effective in experimental trials carried out in New Zealand, Australia, China, Argentina, Chile, Romania and Iran (Lightowlers et al., 1999; Heath et al., 2003; Lightowlers, 2006, 2012).

Larrieu et al. (2013, 2015) undertook a field trial of the vaccine in a remote region of Rio Negro Province of Argentina. Despite the difficulties of working in the region and a large proportion of the animals not receiving their full vaccination compliment, a significant reduction in *E. granulosus* infection was observed in 5-year-old animals vaccinated initially as lambs. To minimize the number of interventions required to be undertaken in the animals, lambs were vaccinated with two injections approximately one month apart and a single subsequent booster injection was given when the animals were approximately 1 year of age. This regime appears to have been sufficient to induce protection lasting until the animals were assessed for infection, at 5 years of age (Larrieu et al., 2015).

Mathematical modelling of various *E. granulosus* control options (Torgerson and Heath, 2003) suggests that a combination of vaccination with EG95 together with 6-monthly treatment of dogs with PZQ would provide an effective strategy for achieving a rapid and high level of control of CE transmission.

The EG95 vaccine has been registered for use in China for some years, although there has been no full publication of data relating to the use or effectiveness of the vaccine manufactured in China. The EG95 vaccine is also registered for use in Argentina. To this time the only published descriptions of the use of EG95 in Argentina have involved vaccine provision at no cost and produced at the University of Melbourne. The Argentine company Tecnovax has recently supplied commercially-produced EG95 vaccine to CE control projects being undertaken in sheep in Chile, however, the high price that was charged for the vaccine (US$ 1.8−1.9 per dose) raises concern. Most countries in which CE is highly endemic are poor countries

and often it is the poorer livestock keepers in these countries that are associated with the highest levels of CE transmission. In order to maximize the potential for EG95 to reduce CE transmission in such circumstances, the vaccine must be made available at a minimum cost. Ideally, it could be incorporated in other, commercially important vaccines such as clostridial vaccines, at little or no additional cost to the livestock owner.

2.3.3 Hydatid anthelmintics for livestock

Benzimidazole-based chemotherapy used frequently for medical treatment of CE in humans involves patients taking the drug on a daily basis over extended periods (Brunetti and Junghanss, 2009). Such treatments are not suitable for routine treatment of CE in livestock animals. If a practical and effective drug treatment could be developed for livestock animals that involved a single treatment, or a small number of anthelmintic treatments, which rendered CE cysts either non-viable or at least, non-fertile, this would provide a significant advance for the control of CE transmission.

Several studies have investigated the effects of various anthelmintic treatments on hydatid cysts in naturally infected animals (Heath and Lawrence, 1978; Gemmell et al., 1981; Schantz et al., 1982; Morris et al., 1985, 1990; Blanton et al., 1998). The primary emphasis of many of these studies was to use hydatid disease in sheep (and goats in one study) as models for testing chemotherapy regimens that could be suitable for use in humans. Many studies found that chemotherapy regimens in which animals received drug doses at daily or weekly intervals over an extended period did achieve the death of cysts, however, this is of limited significance in relation to the control of CE transmission. Heath and Lawrence (1978) and Gemmell et al. (1981) investigated the use of mebendazole or PZQ specifically for the purpose of treating infections in sheep and included either single treatments or relatively short treatment regimes. However, neither was able to demonstrate any adverse effect on cysts.

Several, more recent attempts to treat CE cysts in sheep have met with partial success (Dueger et al., 1999; Mitrea et al., 2007; Gavidia et al., 2009, 2010), although all have involved multiple treatments of the animals at weekly or more frequent intervals. The most effective treatment identified to date, daily dosing at 30 mg of oxfendazole per kg, was found to be unacceptably toxic, resulting in a 24% death rate for treated sheep (Dueger et al., 1999). At this time there is no effective, practical method available for chemotherapy of CE in livestock animals that could be implemented as part of a CE control programme.

2.3.4 Livestock management

It is well established that in endemic areas of echinococcosis, the prevalence and abundance of hydatid cysts increase with the age of the livestock. This has been shown for a number of species including sheep and goats (Gemmell, 1990; Ming et al., 1992; Cabrera et al., 1995; Torgerson et al., 1998), cattle (Torgerson et al., 2003a,b; Lahmar et al., 2013), camels (Lahmar et al., 2013, 2004) and donkeys (Mukbel et al., 2000; Lahmar et al., 2014). In addition the cyst size and fertility increase with the age of animal (Torgerson et al., 2009). This means that most of the infective parasite biomass is in the oldest animals in the population and it is these animals that pose the greatest risk of infection to dogs. This is a particular issue if meat is not the primary product as animals specifically bred for meat production are often slaughtered young. In Tibetan communities there is a religious prohibition to killing animals and most stock are kept for wool or milk and thus some animals reach considerable longevity before dying of natural causes. In India cattle and buffalo appear to have the highest prevalence of echinococcosis amongst domestic animals (Pednekar et al., 2009) and are kept for dairy as beef is not eaten mainly by the Hindu communities. In Uttar Pradesh buffaloes have been reported to have a prevalence of 36% with a cyst fertility rate of 15% compared to a prevalence in 2% sheep and goats with a fertility rate of just 2% (Irshadullah et al., 1989). India has a high burden of CE, possibly second only to China (Torgerson et al., 2015).

This age-related abundance of infection could potentially be exploited. In a study in Kyrgyzstan, it was shown that old sheep (≥4 years) had 80% of the protoscoleces but represented just 28% of sheep presenting for slaughter (Torgerson et al., 2009). These observations on the dynamics of the infective biomass in intermediates hosts suggest that management of the livestock population by removing old animals (such as through culling) would lead to a massive and rapid reduction in the infection pressure to dogs and could be a powerful tool to control CE. This has not yet been attempted in practise.

2.4 Modelling transmission of *Echinococcus granulosus*

2.4.1 Transmission dynamics

Mathematical models of the life cycle were first developed by Roberts and co workers and by Harris and co workers in the 1980s (Harris et al., 1980; Roberts et al., 1986). The model developed by Roberts et al. has now been applied and developed further in various definitive and intermediate host

populations (Gemmell, 1990; Ming et al., 1992; Cabrera et al., 1995; Torgerson et al., 1998, 2003a,b; Torgerson and Heath, 2003; Torgerson, 2006a). This model has both quantitative and qualitative forms and models the changes in parasite abundance and prevalence with age in either intermediate or definitive hosts. Important parameters include the death rate of the parasite, the possibility of acquisition of parasite-induced immunity and the prevailing infection pressure or exposure rate. In the intermediate host the numbers of cysts in infected animals increase with age (which is a proxy for time) and the prevalence approaches an asymptote of one in the oldest animals. This appears to hold true for every intermediate host investigated including sheep, goats, cattle, camels and donkeys (Gemmell, 1990; Ming et al., 1992; Cabrera et al., 1995; Torgerson et al., 1998, 2003a,b; Mukbel et al., 2000; Lahmar et al., 2004, 2013, 2014). This model, therefore, provides a straightforward means to estimating the infection pressure to a group of animals. Representative groups of animals from each age group are necropsied, their cysts counted and the data fitted to the model. Knowledge of the infection pressure to the intermediate hosts can give a strong indication of the force of control efforts needed to control and eventually eliminate the parasite. The model also shows that it is essential to record the ages of animals during surveillance studies. If only young sheep, perhaps, slaughtered in an abattoir close to a city are used it can give a mistaken impression of a low prevalence of echinococcosis in the sheep population.

The model has also been applied in dog populations using arecoline surveillance or necropsy data (Lahmar et al., 2001; Torgerson et al., 2003a,b; Budke et al., 2005b). In dogs the model can estimate reinfection rates (number of exposures per year) that would be useful when deciding the frequency of anthelmintic treatment of dogs during a control programme. The model also indicates the possibility that there is parasite-induced protective immunity in dogs against reinfection as old animals often have lower parasite abundances than young animals. However, this might also be explained by different behaviours in young and old dogs resulting in differences in infection pressure to the two age groups. Mathematical analysis of data sets suggests that there is difficulty in differentiating between the two scenarios (Torgerson, 2006b). This is arguably an important question to answer as good herd immunity in dogs could pave the way for future vaccine development. However, experimental vaccine studies in dogs have so far been unconvincing with regard to possible vaccine development (Torgerson, 2009) (see section 2.2.4).

2.4.2 Optimizing interventions

Having fitted suitable surveillance data from definitive and intermediate hosts to the model and obtaining estimates of the infection pressure or frequency of infection, it is then possible to model the possible outcomes of a control intervention (Torgerson, 2003). Using PZQ to treat dogs can be modelled, for example, as decreasing the life expectancy of the parasite or the use of the sheep vaccine might be modelled as a decrease in infection pressure to dogs. This is reviewed in Torgerson and Budke (2003) and Torgerson and Heath (2003). This model has suggested that, in most instances, treatment of dogs every 3 months with PZQ will effectively control echinococcosis provided the majority of dogs are treated. If sheep are also vaccinated, it may be possible to reduce treatment frequency to every six months.

The model has also been developed further to model the infectious biomass in sheep – that is in terms of protoscoleces per sheep rather than abundance or prevalence of cysts (Torgerson et al., 2009). Using a data set in Kyrgyzstan where the prevalence in sheep is approximately 64%, it demonstrated that 80% of the infectious biomass was in sheep ≥4 years of age, although these animals were just 28% of animals slaughtered. This, therefore, provided a rationale that targeted control or culling of old sheep could be a potentially effective way of immediately substantially reducing infection pressure to dogs and shorten the time period to control.

2.5 Health education

Health education can play a vital role in reducing transmission of echinococcosis to humans. The first successful elimination campaign of *E. granulosus* was undertaken in Iceland. Initially the programme relied heavily on education. In 1864 a 16-page booklet was published in Icelandic, emphasizing the need to destroy cysts and cleaning dogs of tapeworms using areca extracts. This was delivered free of charge to every household. There was a scarcity of books in Icelandic so the booklet was read, possibly by everyone as the population was highly literate (Beard, 1973). The prevalence of CE in the Icelandic population, as shown by autopsy was over 20% in the latter part of the 19th century. But by the middle of the 20th century the parasite had been eliminated (Beard, 1973). The situation in Iceland was, perhaps, the ideal and unique because the population was literate and highly receptive to educational messages.

Echinococcosis is often highly endemic in underdeveloped or resource-poor communities where education is inadequate and there are high levels of illiteracy (Ito et al., 2003b). In an endemic area of Morocco only 50% of

people have heard of the disease, and of those, only 21% were aware of the role of dogs in disease transmission (El Berbri et al., 2015). Likewise in an endemic area of Turkey, 84% of the population had no knowledge of the disease (Ertabaklar et al., 2012). In Tunisia, the highest incidence of human CE was found in areas with the lowest rates of literacy (Chahed et al., 2010). Even medical professionals may have poor knowledge of the disease. In both urban and rural health facilities in Tanzania, only a few practitioners were observed to have the correct knowledge on the transmission of echinococcosis (John et al., 2008).

Other than in Iceland the potential benefits of education have been demonstrated in several epidemiological studies. In the Kyrgyz republic it has also been shown that dogs from households where there was some knowledge of echinococcosis had a lower coproantigen positivity than those from households with no knowledge (Mastin et al., 2015). A similar phenomenon has also been observed amongst the Turkana in northern Kenya (Buishi et al., 2006) and in Libya (Buishi et al., 2005a). However, introducing educational materials into the population can be a challenge where there are low levels of literacy. For example, on the Tibetan plateau education of dog owners and their children about hydatid control was only partly achieved during a 5 year period and was hampered by a high proportion of illiteracy with few children going to school. Eventually a laminated colour page illustrated by cartoons was distributed to most households (Heath et al., 2006). By contrast in New Zealand nearly 30 years of specific health education failed to significantly impact on human or animal infection rates (Gemmell et al., 2001).

Echinococcosis is also a food-borne disease with transmission to humans by contaminated vegetables (Torgerson et al., 2015). Thus in endemic areas, it is essential that dogs are kept off kitchen gardens and fresh produce is well washed to avoid infection of humans by this indirect route. Studies by (Shaikenov et al., 2004) found 5 of 120 soil samples taken from gardens of rural homesteads in southern Kazakhstan were contaminated with *E. granulosus* (G1 strain) eggs.

2.6 Integrated control for cystic echinococcosis

A number of successful CE control programmes have relied heavily on a single tool, such as education in Iceland (Beard, 1973), or through dog control and treatment as in New Zealand (Gemmell, 1990), Cyprus (Economides and Christofi, 2000) and Tasmania (Jenkins, 2005). Whilst they have been successful, they have often required several decades of

sustained effort to bring about effective control and elimination. They are also costly and require a high compliance rate to be successful and there may be difficulty in treating stray dogs. Integrated control uses not only anthelmintic treatment of dogs and dog population control, but also incorporates other control methods such as the use of the EG95 livestock vaccine, education and the control of slaughter of domestic animals. It may also be extended to include the control of other zoonoses or animal diseases such as vaccination of dogs against rabies or vaccination of sheep against brucellosis; both brucellosis and rabies often occur in *Echinococcus*-endemic regions.

Modelling has suggested that the use of both livestock vaccination and treatment of dogs could reduce the frequency of anthelmintic treatment of dogs that is required whilst still achieving effective control (Torgerson and Heath, 2003). Thus such a programme might consist of vaccination of all young animals (twice), one annual booster immunization of all previously vaccinated livestock and six-monthly treatment of all dogs with PZQ. This would save considerable amounts of resources in terms of logistics and costs. The effectiveness is, at least in theory, because there is intervention in both life cycle hosts that has a potentiating effect and thus means compliance rates can be lower to result in effective control. Although the vaccine is now being assessed and used in China, Argentina and Chile and likely to be used elsewhere, there are, to date, no data to confirm the theoretical results of modelling.

If this integrated vaccination of sheep and anthelmintic treatment of dogs was further combined with education and improvement of slaughter facilities, control of CE would be further facilitated. Improvement of slaughter facilities ensures that animals are slaughtered under veterinary supervision, and this should improve the safe disposal of offal to interrupt the disease cycle. Slaughterhouses provide opportunities for surveillance of echinococcosis and other diseases; however, such facilities are found in only a few endemic areas. Modern slaughterhouses are usually expensive, but building low-cost concrete-slab buildings in remote areas is a viable alternative (WHO, 2011). Educational materials should be produced and distributed to the population. These materials should be easy to understand, particularly since there may be a high level of illiteracy, and they should be culturally relevant to the target population (e.g., Heath et al., 2006).

Finally the culling and hence safe removal of old and likely infected livestock animals from the population should be considered. As discussed above this would have the advantage of immediately reducing the infection pressure to dogs considerably.

3. SURVEILLANCE FOR *ECHINOCOCCUS GRANULOSUS*

3.1 Surveillance of cystic echinococcosis in humans

Surveillance in humans is critical for a decision to embark on a CE control programme and also to inform the public and the control authority about the progress of control. Surgical and medical treatment data have been the key to measuring the public health impact and burden of CE disease in endemic communities (Schantz, 1997; WHO/OIE, 2001; Budke et al., 2006). In addition, community screening using ultrasound scanners for abdominal CE case finding has more recently been adopted to provide epidemiological and surveillance data (Macpherson et al., 2003). Serological surveys have a more limited use in surveillance and should not be used as a primary screening tool.

3.1.1 Hospital records

Annual incidence per 100,000 population is the most commonly used index for the frequency rate of human CE at district, provincial or national level. Such hospital data are usually based on surgical case rates (primarily abdominal and thoracic) but should also include cases treated medically (i.e., by benzimidazole chemotherapy), and also those CE cases that are confirmed but not treated (i.e., under 'watch and wait' management). Age-specific incidence especially for the <15 years group can provide the most relevant 'recent' data (as opposed to old cases contracted preintervention) in relation to efficacy of an extended hydatid control programme. Hospital-based retrospective and prospective data sets can indicate the success of control measures or conversely the failure or poor efficacy of interventions, though that may not be very clear until 5–10 years after the start of a CE control programme. Hospital records may not, however, be accurate for the total number of CE cases treated because of poor access to affordable treatment, underreporting, case spread over separate surgical specialities, misdiagnosis and poor record keeping (Craig et al., 2007a). Furthermore, asymptomatic cases may not be identified and thus not treated, unless after accidental detection, and thus are usually not recorded.

3.1.2 Active mass screening for human cystic echinococcosis

Active screening via radiographic surveys (ultrasound, X-ray) should provide a more accurate measure of total CE cases at community level and include known cases, new cases and asymptomatic cases. This then will provide a community cross-sectional prevalence (%) rather than incidence rate per

year, but could also be used in longitudinal studies when communities are screened annually or multiannually. Age-specific ultrasound prevalences again can be extremely useful in the assessment of the impact of interventions associated with a CE control programme, for example, as shown in Rio Negro, Argentina (Frider et al., 2001) and in Turkana, Kenya (Macpherson and Wachira, 1997). One problem is that although the majority of human CE cases are hepatic, around 10% will be pulmonary and thus not detected in routine ultrasound screening. The parallel use of mobile X-ray units is thus required, but that has only been applied in relatively few screening programmes (e.g., Schantz et al., 2003). Where accurate livestock slaughter data are difficult or impossible to collect, such as in many underdeveloped pastoral regions, then age-specific human CE ultrasound prevalences can provide alternative data to measure control efficacy (Macpherson et al., 1984; Larrieu et al, 2004b; Heath et al., 2006).

Serological-based screening for human CE has been applied in epidemiological settings (see review by Rogan and Craig, 2002), but sensitivity and specificity are not sufficiently robust to warrant sero-testing as a primary screening method. A positive serological result alone is not a diagnosis of CE. Supportive or confirmative use of hydatid serology in conjunction with ultrasound-based screening can have a potentially useful role (e.g., Feng et al., 2010), but even then care is required for interpretation of sero-positive but image-negative persons.

3.2 Surveillance for *Echinococcus granulosus* in dogs

The major hydatid control programmes over the period 1960s—2000s used annual ovine CE prevalence together with annual human CE incidence rates as the primary surveillance data. In addition, for owned dog populations, annual or multiannual arecoline purge data was used for surveillance of canine echinococcosis in almost all programmes including New Zealand, Tasmania, Uruguay and Welsh programmes (Craig and Larrieu, 2006). Necropsy data for unwanted and stray dogs were utilized in the hydatid control schemes in Cyprus and La Rioja (Spain) (Economides et al., 1998; Jimenez et al., 2002). With the advent of coproantigen ELISAs in the 1990s, laboratory testing of dog faeces was possible on a large scale and coprotests were used for surveillance in Wales (Buishi et al., 2005b) and Cyprus (Christofi et al., 2002) after main intervention measures had ceased. Copro-ELISA is currently employed as the main screening test for canine echinococcosis in the Rio Negro (Argentina) (Larrieu et al., 2000a; Morel et al., 2013) and west China (WHO, 2011) hydatid control programmes.

Coproantigen ELISA has effectively replaced arecoline testing as the main screening test for dogs in current hydatid programmes (WHO, 2011; Craig et al., 2015). In addition, the advent of copro-PCR tests from the early 2000s (Abbasi et al., 2003) has provided a valuable tool for the species-specific confirmation of *E. granulosus* infection in dogs for both epidemiological and surveillance studies (reviewed by Craig et al., 2015).

3.2.1 Necropsy of dogs

Necropsy examination of the entire length of the dog small intestine for the presence of *Echinococcus* tapeworms is the gold-standard for detection of canine echinococcosis (Craig, 1997; WHO/OIE, 2001). The obvious drawback for necropsy is that it requires dogs to be euthanized, which may not be acceptable or feasible and might lose support of community participation in hydatid control programmes (Kachani and Heath, 2014). Dogs that might be available for euthanasia are usually either unwanted owned dogs (e.g., old, sick, dangerous) or unowned stray dogs in a district or municipality (e.g., nuisance scavenging, aggressive packs, via rabies control) (see Section 4.1.3). Stray dogs may have a higher or lower exposure risk for *Echinococcus* compared to owned dogs (primary target for surveillance), and thus may not be the most representative sample for canine echinococcosis surveillance. Necropsy data from unwanted or stray dogs has been used to assess prevalence of canine echinocococcosis, for example, in the La Rioja (Spain) hydatid control programme (Jimenez et al., 2002), and for validation of coproantigen-based surveillance of dogs in a hydatid control zone in north-west Xinjiang, China (van Kesteren et al., 2015).

Biohazard considerations are required for necropsy in the field or in a laboratory, and an experienced person is needed to undertake necropsy probably also with supporting technical assistance. Adult *E. granulosus* (3–7 mm) occur in the upper duodenum starting close to the stomach and usually are attached over the first third of the small intestine in dogs. The sedimentation and counting technique (SCT) is the most sensitive technique and thus the most accurate for detection and counting worms (WHO/OIE, 2001). Other postmortem techniques include the more practical field applicable direct examination method wherein short lengths of fresh gut are incubated in warm saline to detach worms, which can be examined/counted in the sediment (Craig et al., 2015). Under low magnification (e.g., hand-lens or low-power microscopy) the characteristic morphology of *E. granulosus s.l.* should be confirmed. In coendemic areas where dogs may be exposed to both *E. granulosus s.l.* and *E. multilocularis*

then differential morphology (position of genital pore, uterus form) of *Echinococcus* worms will be required (Craig et al., 2015).

3.2.2 Arecoline testing for canine echinococcosis

Areca nut extract or the synthetic compound arecoline hydrobromide has been used as an intestinal purgative since the late 19th – early 20th Centuries, effectively both as a crude dewormer (Section 3.1.1) and also for premortem detection of *E. granulosus* in dogs. From the 1960s it was the primary tool for surveillance of canine echinococcosis in hydatid control programmes (Gemmell, 1978). Tens of thousands of rural-owned dogs were screened over 10–20 years using arecoline purgation in all the five 'Island' hydatid programmes (i.e., Iceland, New Zealand, Tasmania, Falkland Islands, Cyprus) (Section 6.1.1) and among others Regions XI and XII in Chile, Neuquen and Rio Negro (Argentina), and Uruguay programmes (Craig and Larrieu, 2006). Arecoline testing still remains useful because of its very high specificity especially in relation to transmission studies and epidemiological studies or precontrol baseline investigations (e.g., Hoida et al., 1998; Budke et al., 2005c; Ziadinov et al., 2008; van Kesteren et al., 2013).

The major drawback with arecoline testing of dogs is the difficult logistics and organization needed to undertake purging on the large scale required for surveillance of a hydatid control programme. Tied dogs usually purge within 30–60min but some take longer, others fail to purge, while some react toxically or become dehydrated from arecoline salts. The sensitivity of arecoline purgation after a single oral dose (2 mg/kg) varies from 40% to around 75% (Lahmar et al., 2007). Microscopic examination of boiled or formalin-fixed purges in the laboratory will usually have greater sensitivity compared to diagnosis in the field (Craig et al., 1995; Gemmell and Schantz, 1997), and greater species specificity in areas coendemic for *E. multilocularis* (Budke et al., 2005c). The potential health educational value of purgation for the dog owners is often significant and beneficial, after owners observe the biohazard clothing and strict conditions undertaken by dosing staff, and intact moving tapeworms (especially the large common *Taenia* spp) voided by treated dogs (Gemmell, 1990; Farias et al., 2004). Detection of *Taenia hydatigena* after arecoline testing has been used in hydatid control surveillance as an indicator of dog access to offal and thus 'flags-up' risk behaviour for transmission of echinococcosis (Economides and Christofi, 2002).

3.2.3 Coproantigen tests for canine echinococcosis

The possibility for detection of specific taeniid tapeworm antigens in canid faecal supernatants (coproantigens) was considered in the 1960s (Babos and

Nemeth, 1962) and more intensively investigated as a diagnostic approach for taeniid cestodes, using capture antibodies in ELISAs, from the late 1980s (Allan and Craig, 1989) and for experimental and natural infections of canine echinococcosis in the early 1990s (Allan et al., 1992; Deplazes et al., 1992). Coproantigen ELISAs that utilize anti-*Echinococcus* progottid somatic (Allan et al., 1992; Pierangeli et al., 2010) or ES (Deplazes et al., 1992; Malgor et al., 1997) capture antibodies purified from rabbit hyperimmune antisera appear to be relatively robust with good genus specificity for *Echinococcus* spp usually >90% and sensitivities >70% (Allan and Craig, 2006; Carmena et al., 2006). Anti-*Echinococcus* monoclonal antibodies have potential advantages over polyclonals (Casaravila et al., 2005; Morel et al., 2013) especially in relation to reagent batch and test standardization, though specificity may not be significantly better, nor detection of low worm burdens. *Echinococcus* worm burdens below 50–100 worms may result in some false negatives, and infections due to *T.hydatigena* have been reported to result in some false positives (Allan et al., 1992; Malgor et al., 1997; Morel et al., 2013). It is important to assess a given coproantigen ELISA (including commercial kits) against a panel of faecal samples from dogs with parasitologically confirmed monospecific infections (i.e., necropsy or arecoline purge of experimental or natural infections) including *E. granulosus* or *Taenia* spp., so that the test parameters are determined prior to local application (Morel et al., 2013; Huang et al., 2013; Craig et al., 2015).

Overall the levels of test sensitivity and specificity of coproantigen ELISAs achieved by independent groups (see review by Craig et al., 2015) are at least comparable to arecoline purgation and thus has lead to recommendations that copro-ELISA has the potential to replace arecoline testing as a diagnostic test for canine echinococcosis (Craig et al., 1995; Gemmell and Schantz, 1997; Guarnera et al., 2000; WHO/OIE, 2001; Lopera et al., 2003; Pierangeli et al., 2010; Lembo et al., 2013; Morel et al., 2013).

3.2.3.1 Coproantigen screening in cystic echinococcosis control programmes

Coproantigen ELISAs have now been employed in several hydatid intervention/control programmes to determine baseline levels of canine echinococcosis and/or for surveillance in place of (previous use) arecoline testing.

In mid-Wales (UK) arecoline testing was initially used to determine precontrol levels (prevalence 4.6–25%) of canine echinococcosis in farm dogs prior to 1983 (Craig and Larrieu, 2006) before a supervised six weekly

PZQ dosing programme was implemented over a 6-year-period (1983–89). A coproantigen ELISA developed in 1992 (Allan et al., 1992) was subsequently used in 1993 to screen a sample of farm dogs in the Welsh intervention zone, and recorded 0% copro-positives versus 2.4–9.2% in neighbouring nonintervention zones (Palmer et al., 1996). Dog-dosing measures were terminated in 1989 for economic reasons, and subsequent follow-up copro-ELISA surveys in 2002 and 2008 recorded 8.5% and 10.6% copro-prevalences in farm dogs in the intervention zone (Buishi et al., 2005b; Mastin et al., 2011). In 2010–11, three monthly PZQ dosing (i.e., four times per year) was reintroduced in the original intervention zone for a pilot period of one year only. Coproantigen testing indicated that after 3–4 months copro-prevalence in farm dogs had reduced in the intervention zone from 8.8% to 1.9% and further to 0% in the last quarter, i.e., 12 months after the first dosing round. However, copro-prevalence in farm dogs started to rebound in the following 12 months after dosing had stopped (Lembo et al., 2013).

In Cyprus after the highly successful hydatid control programme in the 1970s (elimination declared in 1985), most of the island was under a maintenance phase of control based on CE surveillance (abattoirs, hospitals). In addition, over the period 1997–2000 more than 6500 owned dogs were screened using coproantigen ELISA, of which 2.8% were copro-positive and treated with PZQ (Christofi et al., 2002).

On the Falkland Islands (Malvinas), since the late 1970s there has been an ongoing government-managed PZQ dosing programme (currently by owners) for all dogs (estimated 900–1000). In 1992–3 coproantigen testing of 464 dogs on the Islands detected 1.7% copro-positives (Reichel et al., 1996). Further testing (n = 563 dogs) in 2010 indicated that coproantigen prevalence had reduced to <0.1% (Lembo et al., 2013). This low coproantigen prevalence in dogs, coupled with very low ovine CE prevalence (<0.02%) suggested that transmission of *E. granulosus* is close to elimination from the Islands and is not now a public health problem (last human case 1992). Thus dog dosing could be made voluntary and a decision made to transfer to a permanent consolidation phase with sole reliance on abattoir surveillance, farm trace-back, quarantine measures and targeted dosing of dogs (S. Pointing, personal communication).

Hydatid disease control in several provinces in the Patagonia region of Argentina has been ongoing since the 1970s, and coproantigen ELISA has been applied to screen dogs in the Rio Negro and Neuquen programmes. In the latter, coproantigen prevalence was 12.4% (n = 403 dogs) compared

to 3.7% based on arecoline testing. An 'in house' coproELISA was used with 93.6% sensitivity and 88.5% specificity (Pierangeli et al., 2010). CoproELISA was used as a farm dog surveillance test to compare sheep ranches in hydatid control zones in Patagonia, which indicated district copro-prevalences from 2.9% (Rio Negro) to 13.9% (Tierra del Fuego), and overall 7.3% of 352 farms sampled in Neuquen had a copro-positive dog (Cavagion et al., 2005). In Rio Grande do Sul, the main CE endemic region of south Brazil where hydatid control was considered, a coproELISA baseline prevalence of 27.7% was obtained, which reduced to 0% copro-prevalence 30 days after PZQ dosing. However, 4 months posttreatment copro-prevalence was 47.4%, which suggested to the authors (despite a small sample size) that dosing frequency should be every 30 days (Farias et al., 2004).

An investigation of canine echinococcosis using a coproELISA was undertaken in 2002 in the remote northwest Turkana district of Kenya 5 years after the effective dismantling of a hydatid control programme which ran from 1983 to 1997 (Macpherson and Wachira, 1997). In the intervention zone, 29% of owned dogs were copro-positive, and furthermore 33% of necropsied unwanted dogs were infected (Buishi et al., 2006). Clearly transmission to dogs was still occurring, however, mean worm intensity was 53 worms per dog in the intervention area compared to 1416 worms per dog in an area not covered in the previous control programme. This suggested that the intensity of transmission had remained low in the intervention zone (Buishi et al., 2006). This result also indicated the usefulness of *Echinococcus* worm burden estimates, which can only be obtained from necropsy or arecoline purgation studies.

In northern Ganze Tibetan Autonomous Prefecture (Sichuan Province, China) a 5 year (2000–05) echinococcosis pilot control programme incorporated six monthly PZQ dosing of owned dogs and applied a coproantigen ELISA for canine surveillance. Copro-prevalence in owned dogs reduced from a precontrol baseline of 50% to 17% after 5 years (Heath et al., 2006; Yang et al., 2009). This high altitude remote area was difficult to reach more than twice per year mainly because of seasonal limitations; however, the coproantigen results suggested that a more intensive dosing pressure would be required together with removal or dosing of the large stray dog population (Heath et al., 2006). Since 2006–07 a national echinococcosis control programme has been rolled out across western China (including Ganze) with target monthly dog dosing and the coproantigen testing of dogs as a key surveillance tool (Huang et al., 2013). In a remote Mongolian

autonomous county (Hobukesar) in northern Xinjiang (China) also subject to this national programme, an independent evaluation of dog treatment and surveillance, gave a copro-prevalence range of 15–70% over six communities investigated by van Kesteren et al. (2015). Furthermore, household questionnaire data indicated significant variation in dog-dosing practice and supervisory visits by veterinary auxillaries (van Kesteren et al., 2015).

These few examples indicate the potential usefulness of coproantigen surveillance in connection with hydatid control programmes; however, limitations include test availability (especially commercial kits), variable test sensitivity and specificity and lack of indication for worm burdens.

3.2.4 Copro-PCR tests for canine echinococcosis

The application of the polymerase chain reaction to amplify *Echinococcus* spp DNA extracted from adult worms, egg concentrates, or whole faecal extracts, provided for the first time the potential for laboratory-based antemortem species-specific identification of canine or vulpine echinococcosis (Bretagne et al., 1993; Mathis et al., 1996; Cabrera et al., 2002a). The development of a copro-PCR test for the detection of *E. granulosus s.l.* DNA directly in canid faecal samples that gave 100% specificity versus *E. multilocularis* and *Taenia* spp (Abbasi et al., 2003), provided the potential to equal or improve the parasitological specificity of arecoline testing. PCR testing for canine echinococcosis has been applied in CE endemic areas (*E. granulosus s.l.*) and in CE/AE coendemic areas (i.e., both *E. granulosus* and *E. multilocularis* transmission) in Kazakhstan (Stefanic et al., 2004), western China (Moss et al., 2013) and Kyrgyzstan (van Kesteren et al., 2013). The sensitivity for *E. granulosus* egg-equivalent detection ranged from one to four eggs per gram of faeces (Abassi et al., 2003; Boufana et al., 2013) and DNA was detectable in faeces from prepatent infections with *E. granulosus* by 21–25 days post infection (Lahmar et al., 2007).

At least 10 copro-PCRs had been published by 2015 for detection of *E. granulosus s.l.* infection in dogs by targeting various genes or repeat elements (i.e., *cox1, 12sRNA, NAD1, EgG1HaeIII*) that are specific for *E. granulosus s.l.* but varied in test genotypic specificity (reviewed by Craig et al., 2015). In the absence, or more likely unwillingness, to undertake necropsy or purgation of dogs in endemic areas for baseline studies and surveillance, then PCR should provide the only unequivocal method for specific confirmation of *E. granulosus* infection via DNA detection. However, PCR is a relatively complex and expensive procedure requiring good laboratory facilities, and therefore has to date had limited routine

application in hydatid control programmes. Therefore copro-PCR in current form, or the lower-tech loop-mediated isothermal amplification methods (Salant et al., 2012; Ni et al., 2014), is not yet recommended as a primary screening tool for canine echinococcosis. The ability of copro-PCR, however, to confirm infection by screening a proportion of dogs (e.g., coproantigen positives) can provide supporting data and confidence in the specificity of epidemiological and surveillance data for canine echinococcosis especially in low CE endemic or reemerging transmission areas (Jenkins et al., 2014) or coendemic areas (Stefanic et al., 2004; Boufana et al., 2013).

3.3 Surveillance for cystic echinococcosis in livestock

3.3.1 Meat inspection

Surveillance in abattoirs provides a valuable opportunity for surveillance in livestock, regardless of species of interest. Ideally, for surveillance purposes livers and lungs presented at the abattoir should be visually inspected for larger cysts and palpated for smaller cysts. It may be necessary to slice the liver to enumerate cysts especially in young animals. Both prevalence and abundance of hydatid cysts increases with age (Gemmell, 1990) and so it is essential to record the ages of the animals during any surveillance study. Studies that primarily inspect young animals such as 1-year-old lambs that are slaughtered for meat will underestimate the prevalence of CE and the results of such studies should be adjusted accordingly. Likewise in any longitudinal study to monitor a control programme, for example, similar age groups of animals must be compared at different time points.

3.3.2 Serology for cystic echinococcosis in livestock

Serology as a clinical diagnostic test for CE in humans is highly effective, with most patients infected with viable cysts being unequivocally positive in different types of test and using a variety of *E. granulosus* antigens (Kagan, 1968; Manzano-Roman et al., 2015). Many publications have investigated serological tests for diagnosis of CE in animals, particularly sheep. The results of these studies have been variable; some publications have described high levels of sensitivity and specificity for the tests that were developed (reviewed by Lightowlers, 1990; Craig et al., 2015). These publications must, however, be interpreted with caution.

Humans are relatively rarely infected with cestode parasites. A relatively small number of cestode species are known to infect humans. The situation with livestock animals is very different, especially for sheep and goats. Infections with cestode parasites other than *E. granulosus* are virtually ubiquitous

in sheep and goats, especially *T. hydatigena*, but also other cestode infections are common (*Monezia, Taenia ovis, Taenia multiceps*). There is substantial evidence for antigenic cross-reactivity between the different taeniid species (Yong et al., 1978; Yong and Heath, 1979; Rickard and Williams, 1982; Gemmell et al., 1986a,b; Lightowlers et al., 1993). Possible infections with, or exposure to, cestode species other than *E. granulosus* are vital factors that must be taken into account in any evaluation of serological tests for CE in livestock. Another critical aspect is the age of the animals that are being compared with respect to their responses in serological tests for CE. Livestock animals are likely to accumulate exposure to cestode parasites over their lifespan and potentially have an increased 'background' reaction in serological tests. It is insufficient to compare samples from *E. granulosus* infected animals and uninfected animals or animals infected with other cestode parasites without also taking into account the age of the animals. For example, comparison of samples from aged sheep infected with *E. granulosus* with samples from lambs infected with *T. hydatigena* would not provide a reliable measure of the specificity of a test.

Few studies have taken these factors into account in their descriptions of serological tests for CE in livestock animals. An ideal comparison is the level of serological reactivity in *E. granulosus* infected animals and noninfected animals of the same age and derived from the same flock. When such comparisons have been made, or when comparisons have been made using sera from sheep experimentally infected with various cestodes, high levels of positive responses have typically been seen in animals which have had no *E. granulosus* infection (Lightowlers et al., 1984; Yong et al., 1984; Dueger et al., 2003).

An exhaustive investigation was undertaken of serological reactivity to various *E. granulosus* antigen preparations by Lightowlers et al. (1984) involving sheep serum samples from *E. granulosus* infected and uninfected sheep from the same flock, animals with heavy, large fertile hydatid cysts, and mature sheep from an island where dogs are never present. Differences in the average reactivity of the groups of sera in ELISA were evident between infected flocks and uninfected flocks, however, it was not possible to diagnose reliably the presence of CE on an individual animal basis, even in cases of very heavily infected animals.

Data from serial bleeds of sheep experimentally infected with *E. granulosus* indicate unequivocally that they do respond to the infection, producing specific antibody (Yong and Heath, 1979; Conder et al., 1980; Yong et al., 1984). The level (titre) and frequency of detectable responses (sensitivity),

however, seem substantially different to the titres commonly detected in humans undergoing serological diagnosis for CE. This lead Lightowlers et al. (1986) to investigate the hypothesis that *E. granulosus*–infected sheep may develop a degree of nonresponsiveness to hydatid antigens. A small amount to cyst fluid from the animals' own hydatid cysts was released into the peritoneal cavity at laparotomy. Classic, high-level, anamnestic antibody responses were elicited, indicating that the animals were both primed to respond to hydatid antigens and were not suppressed in their ability to respond, at least insofar as it involved exposure to antigen via this route.

In conclusion, there is currently no serological method that can be used to reliably and specifically diagnose hydatid infection in livestock animals.

3.3.3 Ultrasound for cystic echinococcosis detection in sheep and goats

CE in livestock animals is essentially asymptomatic and therefore premortem clinical diagnosis is not possible. Serological tests remain unusable for routine practical application (see Section 3.3.2). Radiographic methods for cyst imaging, especially portable ultrasound scanners, offer a more practical and reliable approach for the potential premortem diagnosis of CE in small ruminants (see review Craig et al., 2015). Two large studies in 300 Kenyan sheep/goats (Sage et al., 1998) and 120 sheep in Sardinia (Dore et al., 2014) that were scanned then inspected postmortem, indicated that identification of CE–infected sheep or goats was possible in standing animals with sensitivities of 54.4% and 88.7% in respective studies. The calculated specificity for CE was higher (97.6%) in the Kenyan study than in the Sardinian assessment (75.9%), but that might reflect the higher resolution transducer and imager used in the more recent Sardinian study (Dore et al., 2014). The main cause of false positive images in both studies was the presence of cysts of *T. hydatigena,* which had also been noted in another study (Maxson et al., 1996). Certainly the few studies, published on the application of ultrasound for CE diagnosis in sheep and goats, suggest that this approach has good potential and is overall more reliable than current serological tests. In underdeveloped endemic regions where it is difficult or impossible to use meat-inspection data for CE surveillance, then mass ultrasound scanning in live sheep or goats may offer a reasonable alternative.

3.3.4 Sentinel animals

Few, if any, studies have deliberately distributed *known* noninfected animals to act as sentinels for transmission of *E. granulosus*. Logically, all newborn

livestock are effectively sentinels by which continuing transmission can be determined. Lloyd et al. (1991, 1998) described the use of sentinel lambs in mid-Wales for monitoring transmission of *E. granulosus*. The lambs were purchased from their farm of origin and slaughtered at the time of the researchers' choosing. This method may provide a greater assurance that animals would be available for assessment when they were needed, rather than relying on farmers' agreeing to sell animals for necropsy without their having been a preexisting arrangement. In the Welsh study, 6% of sentinel lambs became infected with *E. granulosus* within 19 months of exposure in an intervention area previously under a six weekly dog-dosing regime (Lloyd et al., 1991, 1998). Another study in Uruguay observed that sentinel lambs were not infected at postmortem when six weekly dog dosing was used, but 4–18% of lambs were infected when the dog-dosing frequency was reduced to 12–16 week intervals (Cabrera et al., 2002b).

4. CRITICAL APPRAISAL OF CYSTIC ECHINOCOCCOSIS CONTROL PROGRAMMES

4.1 Successful hydatid control programmes

Since the first hydatid control programme was implemented in Iceland in the 1860s at least 18 other intervention programmes have been undertaken in different world regions to reduce the transmission of *E. granulosus* and to try and reduce or eliminate human CE as a public health problem. Many of these programmes have already been reviewed elsewhere (Gemmell, 1978, 1990; Gemmell and Schantz, 1997; Economides et al., 1998; Gemmell and Roberts, 1998; Gemmell et al., 2001; Craig and Larrieu, 2006; Larrieu and Zanini, 2012; Lightowlers, 2012).

The ultimately successful control programme in Iceland lasted >100 years and eliminated transmission from the island by the 1950s–60s. However, incidence of human CE in the <40 year age group had already fallen significantly by the decade 1890–1900 (Beard et al., 2001). This was in many ways a unique situation where a small, highly literate population responded positively to health education messages and legislation to stop home slaughter, reduce dog contacts and accept annual arecoline treatment of dogs; in addition a national change in sheep husbandry from milk and wool production to marketing fat lamb, helped to reduce the parasite reservoir of ovine echinococcosis (Dungal, 1957; Craig and Larrieu, 2006). The Icelandic success against hydatidosis influenced New Zealand

to begin a health education progamme from 1938 to 1958, which also included free provision of arecoline to dog owners, and legislation making it illegal to feed dogs raw offal. In contrast to Iceland, however, surveillance data from hospitals and abattoirs indicated no reduction in transmission of CE after 20 years of a hydatid educational campaign (Gemmell, 1990). Other health education campaigns that similarly included encouragement of owners to dose dogs, did not appear to have any significant effect on transmission of *E. granulosus* in mid-Wales (after 1989) nor in Sardinia (1969–90) (Craig and Larrieu, 2006).

Replacing long-term horizontal programmes, (where emphasis was on education, abattoir upgrades, meat inspection and dog management), with faster-track vertical programmes based on regular dog dosing (once PZQ became readily available in the late 1970s–early 1980s) was the key to potential success for the majority of hydatid control programmes. Reduction in the adult worm biomass in rural dog populations would relatively rapidly reduce infective pressure to sheep (and other livestock) and humans. Presence of hydatid cysts in older sheep (>5 years) would require application of dog dosing in an attack phase for >5 years and probably 5–10 years. Key aspects of dog dosing with PZQ for a successful outcome were:

- determination of number of owned dogs (registration),
- provide supervised dosing at a frequency of 4–8 times per year,
- dose at least 90% of registered dogs and
- maintain dosing pressure for 5–10 years.

When the above dog-directed measures were applied successfully by a government control authority with a good infrastructure, that included surveillance in sheep, dogs and humans, then prevalence of ovine and canine echinococcosis declined within 5 years and human CE rates within 10 years (Gemmell et al., 2001; Craig and Larrieu, 2006; Larrieu and Zanini, 2012).

4.1.1 Island programmes: New Zealand, Tasmania

In the first half of the 20th century human hydatidosis (CE) was recognized as a major public health problem in rural communities in New Zealand and Tasmania (an island state of Australia) (Lightowlers, 2012). Both territories began vertical-directed control programmes that targeted owned dogs from 1959 to 1964 respectively. The New Zealand programme was initially funded by a dog tax collected by a National Hydatids Council, then after 1991 through the Ministry of Agriculture. The Tasmania programme was funded and implemented from the beginning via the Department of

Agriculture. Both programmes used mass testing of dogs with arecoline, but in New Zealand (from 1978), PZQ was in addition employed to dose dogs at a frequency of 8 times per year (i.e., approximately every six weeks). In Tasmania by contrast, annual arecoline testing of dogs on farms by mobile units was maintained for 11 years (without the use of PZQ) in conjunction with strict quarantine measures for positive farms. Transmission was considered to have almost ceased in Tasmania within 10 years (McConnell and Green, 1979). In contrast, the attack phase in New Zealand using PZQ with centralized testing of purges, lasted 32 years. There were no human CE cases under 20 years of age in Tasmania after 1976, prevalence ovine CE had dropped from 52% to 3.4% by 1978 and dog prevalence to 0.06% by 1985 (Beard et al., 2001).

Tasmania declared the state was provisionally free of hydatidosis in 1996, which is 32 years after the start of the control programme; New Zealand declared itself free of hydatidosis in 2002, a 43 year period after the start (Pharo, 2002; Jenkins, 2005). The Tasmanian hydatid control programme had a shorter attack phase and was very cost-effective utilizing only 0.5% of the state health budget. Part of the success in Tasmania was participation and support of rural communities and the efficient organization of control under the Department of Agriculture, in particular the use of mobile dog testing units for farm outreach and the strict use of enforced quarantine. The examples of hydatid control undertaken by New Zealand and Tasmania were very successful, with the latter declaring elimination of human CE as a public health problem in less than 20 years from initiation. Other hydatid programmes were influenced by the ultimate success of these two Australasian examples (Lightowlers, 2012). What is the current status of transmission in these two regions 15–20 years after declaration of freedom from hydatidosis? Both regions operated effective consolidation phases comprising largely meat inspection and trace-back after control measures had ceased. In New Zealand small lesions in slaughtered livestock were subject to histological examination to rule out CE, while in Tasmania PCR has been employed to confirm lesion identity. In both regions the only human CE cases that occurred postcontrol were in the >40 years age group and thus probably were infected prior to the termination of control measures (O'Hearn and Cooley, 2013). However, complete elimination of *E. granulosus* transmission has probably either not occurred or has reemerged in Tasmania as evidenced by the finding of CE in cattle born on the island and the occurrence of *Echinococcus* PCR-DNA positives in local farm dogs (Jenkins et al., 2014).

4.1.2 Island programmes: Falkland Islands, Cyprus

The incidence of human CE in the small population on the Falkland Islands (Las Malvinas) between 1965 and 1975 was equivalent to 55 per 100,000 per annum (Craig and Larrieu, 2006). Six weekly dog dosing with PZQ was introduced by the Department of Agriculture in 1977. Initially dosing was supervised, then dog owners were expected to dose their dogs with the drug provided free. Surveillance of ovine CE in Stanley abattoir in 1993 indicated a reduction from a precontrol baseline of 59% to 0.16% (Reichel et al., 1996). No preintervention baseline data for canine echinococcosis were available, but coproantigen ELISA indicated 1.7% copro-prevalence in 1993 (Reichel et al., 1996), which had further reduced to <0.1% by 2010 (Lembo et al., 2013). In 2015 dog owners were still expected (via government information and local meetings) to dose their dogs every six weeks with PZQ provided by the Department of Agriculture. In addition an active farm trace-back system was operated, from the single abattoir, to identify and quarantine farms when any hydatid positive sheep were identified by inspection and PCR testing (Lembo et al., 2013). There have been no human CE cases on the Falklands except in the elderly. The Department of Agriculture considers echinococcosis transmission to be on the verge of elimination, however, sporadic occurrence of positive animals (sheep and dogs) persists in isolated farms as well as the continued occurrence of *T. hydatigena* cysts in sheep that indicated probable lack of compliance by owners in relation to dog dosing (S. Pointing, personal communication).

In Cyprus prior to 1971, baseline data on hydatidosis/echinococcosis indicated a human CE incidence of 12.9 per 100,000 per annum, ovine CE prevalence was between 25 and 80% and arecoline purge prevalence of canine echinococcosis was 14% (Economides et al., 1998). Hydatid control was implemented in 1971 by the Department of Veterinary Services (under the Ministry of Agriculture) and largely focussed on active culling of stray dogs, euthanasia for arecoline-positive owned dogs (3 monthly testing) and strict livestock slaughter controls (Polydorou, 1993). Between 1971 and 1985 more than 85,000 dogs were killed. Dog prevalence reduced to 0.75% by 1977 and in 1984–85 none of 36,000 dogs were found infected by arecoline testing. There were no human cases diagnosed in the <20 years age group and hydatid eradication was claimed in 1985 (Polydorou, 1993). Sporadic transmission, however, reemerged between 1993 and 1996 in livestock in 21% of villages and 0.6% of dogs were arecoline test positive in the government controlled area (GCA) of the island (excludes Turkish occupied Northern Cyprus); furthermore, 2.8% of dogs were

coproantigen positive in the period 1997–2000 (Christofi et al., 2002). Control measures were reapplied in the GCA in the mid-1990s which included PZQ dosing of dogs 2–3 times per year, stray dog management (no culling), movement control of livestock and prosecution for illegal slaughtering. Strict quarantine of *Echinococcus*-positive properties was implemented until there was a minimum of 3 years absence of *Echinococcus* or *T.hydatigena* cysts at livestock slaughter inspection (Economides and Christofi, 2002).

4.1.3 South American control programmes: Chile, Argentina, Uruguay

Human CE has been recognized since the mid-20th century as an important public health problem in at least five countries of South America, i.e., Argentina, Chile, Uruguay, Peru and Brazil, with an estimated 2000 cases annually across this region (Larrieu et al., 2004a). Ultrasound mass screening studies in the 1980s and 1990s indicated human CE prevalence in communities ranged from 1.6 to >14% (Moro and Schantz, 2006b). During the period 1970s–90s several vertical control programmes for CE were implemented in Uruguay (nationally) and in regions of the other four countries listed previously. The New Zealand and Tasmania successes were used as models for the South American programmes initially using arecoline then replaced by PZQ as the key dog treatment tool. The aim was to dose dogs eight times per year for at least 5 years in conjunction with health education and appropriate animal and human surveillance (Larrieu and Zanini, 2012). These hydatid control schemes were, however, organized under different authorities, i.e., the Department of Health in Argentina (Neuquen, Rio Negro, Tierra del Fuego) and Peru, the Ministry of Agriculture in Chile (Regions XI and XII) and an honorary hydatid commission in Uruguay. Greatest impacts, as measured by reductions in human incidence or prevalence and sheep and dog prevalences, occurred in Rio Negro (1980–2003), Chile Region XII (1982–97) and Uruguay (1990–2007) (Craig and Larrieu, 2006; Larrieu and Zanini, 2012) (Table 1).

4.1.3.1 Region XII, Chile

In Chile in 1979 the government's Ministerio de Agricultura y Servicio Agricola y Ganadero (SAG) introduced a vertical hydatid control programme for Region XII in the highly endemic south of the country. The main tool in the attack phase was six weekly (eight times per year) supervised dosing of farm dogs with PZQ, which had a significant impact in animal hosts within

5 years. Ovine CE prevalence declined from >60% to 25% and canine echinococcosis from 70% to 5% by 1984. Human CE incidence reduced from >40 per 100,000 to 1.8 per 100,000 per year by 1992 (Gemmell and Roberts, 1998; Craig and Larrieu, 2006). In 1984 dog owners were expected to dose dogs four times per year and the other four times to be carried out by veterinarians. In 1987–88 the dog-dosing frequency was reduced to two times per year to further save costs, however, ovine CE prevalence had plateaued at 5–7% (Vidal et al., 1994; Gemmell and Schantz, 1997). In response SAG reintroduced eight times per year dog dosing in 1991, which drove ovine CE prevalence close to zero by 1994 (Gemmell and Schantz, 1997; Craig and Larrieu, 2006). The transition to a consolidation phase occurred in 1998, which then placed emphasis on voluntary dog dosing and surveillance, however, indications of reemergence of echinococcosis were observed in Region XII by 2002 in sheep and dogs (Alvarez, 2002; Larrieu and Zanini, 2012).

4.1.3.2 Rio Negro, Argentina

The Rio Negro CE control programme in Argentina was launched in 1980 under the Ministry of Health (Provincial Council of Public Health and Department of Zoonoses). Provincial incidence of human CE was 73 per 100,000, and in children 50 per 100,000 (Larrieu et al., 2000a). Health workers were responsible for home visits, distributing PZQ pills to owners and checking on dog-dosing compliance and frequency, while veterinarians undertook surveillance aspects in dogs and sheep. Dogs were dosed four times per year under owner responsibility. Arecoline purge prevalence in dogs was reduced from a 41% baseline in 1980 to 5% by 2008–10. Ovine CE prevalence reduced from 61% to 2.9% by 1998. Importantly, within 12 years of the start of interventions, CE incidence in children (<13 years of age) reduced to below 20 per 100,000; furthermore, CE ultrasound prevalence in children <15 yrs reduced from 5.6% to 0.3% by 2008–10 (Frider et al., 2001; Larrieu et al., 2000a; Larrieu and Zanini, 2012).

The control measures for both the Region XII (Chile) and the Rio Negro (Argentina) hydatid control programmes were applied continuously for more than 15 years in remote resource-poor rural communities. The key measure was dosing dogs with PZQ 4–8 times per year by dedicated technical teams or by effective owner compliance. With upgrade of abattoirs and meat inspection training, surveillance in sheep was able to be efficiently undertaken to provide key surveillance data to monitor progress. CE incidence or prevalence rates in children <15 years indicated that

transmission to humans had reduced significantly in both regions within 10 years (Larrieu et al., 2004b). Both programmes were managed under existing government authorities (Agriculture or Public Health) with good outreach and community acceptance and were also cost-effective, e.g., Rio Negro programme cost US$ 41,000 per annum, which included veterinary and medical costs (Larrieu et al., 2000a). Both programmes transferred from attack to consolidation phases with emphasis on owners to dose dogs and surveillance, abattoir surveillance and trace-back. However, dismantling of the vertical programme (Region XII, Chile) and handover of dosing to farm owners (also in Rio Negro) lead to probable reemergence in Region XII and persistence of low level transmission in Rio Negro (Larrieu and Zanini, 2012).

4.1.3.3 Uruguay

Uruguay was the only country in South America to implement a hydatid control programme at national level. Similar to New Zealand (pre-1990) a national commission against hydatidosis was created in 1965 and funded by a dog tax (Comision Honoraria de Lucha Contra la Hidatidosis). Initially, sheep farm owners were provided with arecoline and expected to treat their own dogs, then later dosing was done by mobile arecoline teams but they probably only covered about 50–60% of rural dogs (Craig et al., 1995). Between 1972 and 85 ovine CE prevalence had not reduced significantly from precontrol baseline of 40–65% and national human incidence was 12.4 per 100,000. In 1992 the programme was relaunched by the Honorary Hydatid Commission under the Ministry of Health, using an enlarged technical team to dose dogs monthly with PZQ, so that by 1995, 90% of farms were being included nationally (Larrieu and Zanini, 2012). Within 5 years (1997) arecoline surveillance indicated a reduced prevalence (0.7%) of canine echinococcosis, and within 10 years ovine CE was <5% in lambs, while incidence of human CE fell to 6.5 per 100,000 (Cabrera et al., 2002b). Thus Uruguay was able to convert a largely unsuccessful control programme, based on a combination of Option 2 (health education) and Option 3 (arecoline testing) type intervention to an effective Option five type intervention scheme based on supervised frequent dog dosing with PZQ. By 2006–07 the Uruguay programme was merged with a new National Commission for Zoonoses under the Ministry of Health and hydatid control moved into a consolidation phase with emphasis on continued monthly dosing, surveillance and dog population management (Larrieu and Zanini, 2012). Coproantigen ELISA was employed to test

dogs and also mass castration and spaying schemes were introduced across the country. Between 2008 and 2013 coproantigen prevalence decreased from 10.2% to 3.4% in rural settlements, and human ultrasound prevalence of CE from 6.5% to 2% with only two CE cases less than 20-year-old (Irabedra et al., 2016).

4.1.3.4 Other less successful South American programmes

Other less successful pilot or regional control programmes in South America were implemented in Neuquen, Argentina (1970), Tierra del Fuego (1976), Peru (1992) and South Brazil (1983). In Neuquen six weekly arecoline dosing and health education were implemented and canine prevalence dropped from 28% to 3% in the first 4 years (Gemmell, 1978). In Tierra del Fuego the aim was to deworm owned dogs every six months and construct dog kennels and slaughterhouses (Zanini et al., 2006). The Peruvian programme centred on the central highlands opted for an arecoline-based approach but was interrupted then halted by political insurgency. An eight month pilot programme in Rio Grande do Sul (Brazil) applied monthly dosing with PZQ and was effective at reducing copro-prevalence in dogs but was not expanded (Farias et al., 2004).

4.2 Eurasian hydatid control programmes

Human CE is endemic in less developed or resource-poor rural zones across large parts of southern and eastern Europe and in contiguous regions across to Central Asia, eastern Russia and China. At least eight formal hydatid control programmes (including Cyprus, see Section 4.1.2) have been funded in this large region, which included schemes in: mid-Wales, UK (1983—89), La Rioja, Spain (1987—2000), Sardinia, Italy (1987—97), Hutubi, Xinjiang, China (1987—94), Datangma, Sichuan, China (2000—2005), Shiqu, Sichuan, China (2006-ongoing) (see Table 1) and a pilot in the Alay Valley, Kyrgyzstan (2011—2015). The Chinese schemes are now under a National Echinococcosis Control Programme to control CE (and AE) in western Provinces and Regions (Zhang et al., 2015). All were set up to include dog dosing with PZQ as the key intervention for a potential fast track approach (i.e., Option 5). One pilot programme (Datangma, China) also included livestock vaccination with EG95 (i.e., Option 6). Primary surveillance was based on prevalence in sheep (abattoir data) and dogs (coproantigen prevalence or necropsy) for the mid-Wales and La Rioja programmes, and on dog copro-prevalence and human ultrasound prevalence for surveillance in the west China national programme.

4.2.1 Europe: Mid-Wales, La Rioja, Sardinia

In both the Powys (mid-Wales) and La Rioja (Spain) hydatid control programmes, supervised dog dosing with PZQ at a frequency of eight times per year (i.e., six weekly intervals) was carried out by government veterinarians continuously for the first 6 years. This resulted in a 90% reduction in canine copro-prevalence (Wales) or dog necropsy prevalence (Spain) and also for ovine CE a reduction of 50–75% for both regions (Palmer et al., 1996; Jimenez et al., 2002). In addition construction of burial pits for safe disposal of sheep carcasses, as well as widespread health education was included in rural communities. The Welsh programme was, however, terminated prematurely after 6 years (in 1989) for economic reasons and replaced by a health education component (i.e., Option 2) that encouraged dog owners to purchase PZQ and to dose their own dogs at least 6–8 times per year (Lloyd et al., 1998; Craig and Larrieu, 2006). However, within 7 years of withdrawal of supervised dosing, coproantigen prevalence in Welsh farm dogs had increased from 0% to 6.3% and was close to 10% by 2002 (Buishi et al., 2005b; Mastin et al., 2011).

A control programme in Sardinia was well funded with extensive health education, but poorly managed (Sardinian Experimental Institute for Zooprophylaxis) with emphasis on owner responsibility to dose dogs. The programme had poor outreach and was not fully accepted by the sheep-raising communities, so that there was no significant reduction in either dog or ovine prevalences over a 10-year-period (Conchedda et al., 2002; Craig and Larrieu, 2006). Home slaughter and poor deworming practices continued in Sardinia (Varcasia et al., 2011). In both La Rioja and Sardinia there were large stray dog populations (80,000 in Sardinia) that were considered an important reservoir of infection. Management of dog populations was difficult and a euthanasia policy was undertaken in northern Spain and an impoundment policy in northern Sardinia, the latter becoming unsustainable.

In the La Rioja, scheme interventions were overall highly successful and included euthanisation of 500–1000 stray dogs per year a proportion of which were necropsied to provide canine echinococcosis prevalence information contributing to the surveillance data, which showed a fall from 7% to 0.2% after 10 years of dog dosing (Jimenez et al., 2002).

4.2.2 China: Hutubi, Datangma, Shiqu

4.2.2.1 Hutubi (Xinjiang)

Xinjiang Uygur Autonomous Region in northwest China is the largest administrative region of the country and remains highly endemic for human

CE (National Hydatid Disease Center of China, 1993; McManus, 2014). A 3-year (1987–90)-pilot control programme was introduced in Hutubi County, mainly Han Chinese area (human CE incidence 43.8 per 100,000) with a mean of 0.86 dogs per household (Andersen et al., 1991). It emphasized supervised monthly deworming of registered dogs using a novel biscuit-baited formulation of PZQ (Chi, 1993), together with stray dog population management and health education (Andersen et al., 1991; Zhang et al., 2009b). Surveillance was based on arecoline purge prevalence in dogs, and ovine CE prevalence in cohorts of purchased sheep. Over a 3 year period canine echinococcosis prevalence was reported to have reduced from 18.5% to 0%, and ovine CE from 88.8% to 5.6%. In total >1200 unwanted dogs were culled and a distemper epidemic in 1988–89 further reduced the dog population by >25% (Zhang et al., 2009b). Community participation was also reported to be excellent even including contribution by dog owners to the cost of dog treatments (US$ 5.2 per year).

The Xinjiang pilot hydatid control scheme in Hutubi in the late 1980s helped to provide evidence that control of CE by efficient registration and dosing of dogs, using Chinese manufactured PZQ, was possible in settled poor livestock-keeping communities in China. As a result that study and others subsequently contributed to the development of a National Hydatid Control Programme launched in 2006 (Zhang et al., 2015). The challenge for the new national programme, however, would be its roll-out and implementation in hard-to-reach semi-nomadic ethnic Tibetan, Kazakh and Mongolian communities dispersed over several provinces in western China. Two such areas were Datangma and Shiqu counties in the Ganze Tibetan Autonomous Prefecture situated on the eastern Tibetan Plateau (3900–5000m altitude) in northwest Sichuan Province, a provincial region where township human CE prevalence (by ultrasound) ranged from 1% to >10% and where human AE disease (<1% to >9%) was also highly coendemic (Li et al., 2010).

4.2.2.2 Datangma (Sichuan)

A pilot intervention scheme, funded by the New Zealand Agency for International Development , was implemented in Datangma County (Ganze Tibetan Autonomous Prefecture, Sichuan Province) in four remote high altitude (>4000m) Tibetan townships (human CE prevalence 0.91–2.61%, human AE 2.59–6.35%) from 2000 to 2005. The pilot comprised twice annual dosing (April and October) of owned (n = >4200 dogs) and stray dogs (~1500) with PZQ, inclusion of a

Tibetan health education programme, and the use of the EG95 vaccine for sheep and goats with annual booster vaccination in autumn (Heath et al., 2006; Yang et al., 2009). Surveillance and monitoring was done by coproantigen testing and also necropsy of a subsample of dogs, serology for EG95 antibodies was carried out in small ruminants, also by meat inspection of purchased cohorts of sheep/goats and 4-year-old yaks. The human population was screened by ultrasound at voluntary clinics, and annual questionnaire surveys were administered to assess positive changes in knowledge and behaviour relating to transmission and hygiene (Heath et al., 2006).

After 5 years of the pilot scheme, the coproantigen prevalence in owned dogs had dropped from 50% to 17%, while necropsy of strays showed a reduction from 63% (includes both *E. granulosus* and *E. multilocularis*) to 36% prevalence. Prevalence in 4-year-old yaks and in <1–6-year-old sheep/goats after 3 years remained high at 38% for both (Yang et al., 2009). Community compliance was, however, not very good and about 25% of Tibetan households did not accept either dog dosing nor livestock vaccination. Serological testing of small ruminants indicated that around 50% had not been vaccinated; furthermore, only around 10% of people attended initial ultrasound screening clinics and even less in subsequent surveys (Heath et al., 2006). This pilot study indicated the great difficulties in applying and sustaining hydatid control measures and effective surveillance in Tibetan communities and also the complication for control in regions where dogs are involved in transmission of both *E. granulosus* and *E. multilocularis* (Lembo et al.,2013).

4.2.2.3 Shiqu (Sichuan), and the China National Programme

From 2006 the Chinese Ministry of Health launched an ambitious echinococcosis control programme at national level, which was initiated in 10 highly endemic counties in northwest Sichuan Province (including Datangma and Shiqu counties) and then extended to 170 counties in 7 provinces/regions (i.e., Sichuan, Qinghai, Gansu, Ningxia, Tibet AR, Xinjiang, Inner Mongolia) (WHO, 2011; Zhang et al., 2015). In Sichuan Province alone there were an estimated 27,000 human echinococcosis cases of which the majority were of Tibetan ethnicity, also livestock CE prevalence was 40–80% (Wang et al., 2008). The main intervention measures proposed were dog deworming using PZQ at monthly intervals and health education including encouragement of community participation. This was accompanied by free or heavily subsidized treatment of human CE and AE cases

(albendazole and/or surgery), community mass ultrasound screening (with serology) and annual coproantigen surveillance in samples of owned dogs. Shiqu County (area 25,000 km^2, mean elevation 4200 m) had a population of approximately 63,000 people (97% Tibetan) with an estimated livestock population (yak, sheep, goats, horses) of 581,000, a dog population of 30,000 (including >4000 strays/community owned) and a mean of 1.34 dogs per family (Budke et al., 2005b; Wang et al., 2006a,b).

In 2002–03 human CE prevalence was 4.9% and AE 6.2% (Tiaoying et al., 2005). Infection rates in dogs by arecoline purgation for *E. granulosus* and *E. multilocularis* were 8% and 12% respectively in 2002–03 (Budke et al., 2005a), and 21% by *Echinococcus* coproantigen ELISA in 2006 (Moss et al., 2013). Although the National Hydatid Programme aimed at monthly dog dosing, this was very difficult to achieve in Shiqu County due to the dispersed Tibetan population and seasonal problems including: severe winters, the mass movement of people, dogs and livestock to summer pastures and springtime activities for traditional medicine collection of 'winter worm' (*Cordyceps sinensis*). Consequently dosing of dogs was aimed at 3–4 times per year (spring, early summer, late autumn and early spring) to be managed by local Centers for Disease Control veterinary technicians. Technicians either carried out supervised dosing or more usually placed reliance on owners to dose their dogs with drug provided free to township dispensaries. After approximately 6years canine copro-prevalence in five townships in Shiqu was reportedly below 1% (Q. Wang, personal communication). The attack phase was ongoing in 2015–16 in Shiqu County with continued evidence of a low copro-prevalence, however, seasonal logistic problems resulted in reduced dog-dosing cover. Human CE and AE age-specific ultrasound prevalence rates are under analysis to help assess any reduction in the younger age groups.

In other areas of China, for example, Hobukesar Mongolian Autonomous County in northwest Xinjiang, the National Control Programme struggled to achieve monthly dosing of owned dogs with some communities reporting only 22% of dogs dosed within six weeks of sampling. Also 41.3% of owned dogs were coproantigen positive and 42% (16/38) of necropsied unwanted dogs were infected with *E. granulosus* (van Kesteren et al., 2015).

4.3 Reasons for success and problematic outcomes in cystic echinococcosis control

Since the 1960s at least 18 hydatid control programmes, schemes or pilots have been carried out or initiated, with periods lasting from 3 to 5 years

to >40 years (see Table 1). The four island programmes that were initiated between the late 1950s and early 1970s, i.e., New Zealand, Tasmania, Falkland Islands and Cyprus, were overall highly successful so that human CE has either been eliminated or is not a significant public health problem in those territories (see reviews by Gemmell, 1990; Gemmell and Roberts, 1998; Craig and Larrieu, 2006; Lightowlers, 2012); furthermore, transmission to humans had virtually ceased within 10 years of the start of vertical interventions (Gemmell et al., 2001). Common enhancing elements in these four programmes were: well structured agricultural sectors, largely literate and compliant rural populations, good veterinary outreach networks, a control authority under a Ministry of Agriculture, sustained ability to undertake supervised dog dosing with PZQ 4–8 times per year (New Zealand, Falklands) or arecoline testing at least once per year with punative quarantine (Tasmania) or euthanasia (Cyprus), efficient local abattoir inspection for surveillance in sheep, good medical data on regional incidence, and ability to transfer from an attack phase (primarily dog dosing) to a consolidation phase (i.e., abattoir surveillance and trace-back). Formal health education components were included in all programmes but appeared to have had little or no direct impact prior to the application of dog-targeted vertical interventions (Gemmell et al., 2001).

4.3.1 South American cystic echinococcosis control programmes

The above features described for the Island programmes were present and contributed to the positive outcome for three large continental-based hydatid control programmes in South America. All had adopted an ‘Option 5’ control approach (i.e., regular dosing of dogs with PZQ), i.e., Rio Negro (Argentina), Region XII (Chile) and Uruguay. Problems caused by a low percentage of dogs treated had slowed initial efforts in Uruguay pre-1990, which was managed by an honorary commission, but became effective when that Commission was restructured under the Ministry of Agriculture (Larrieu and Zanini, 2012). Large reductions in prevalence of ovine CE and canine echinococcosis within 5–6 years in the Region XII (Chile) programme led to a premature relaxation of dog-dosing frequency by the Ministry of Agriculture from eight times per year to four times per year then to twice per year, which resulted in a prevalence plateau in lambs at around 5%. Subsequently dosing was reintroduced to eight times per year that successfully reduced CE prevalence in lambs (Gemmell and Schantz, 1997). Transmission to humans, especially children <15 years old, significantly reduced in Rio Negro (Argentina) but has not been eliminated, in

part due to logistics of dosing dogs eight times per year. A pilot to include sheep vaccination with EG95 was assessed in Rio Negro, and indicated good protection in sheep <5 years old (Larrieu et al., 2015).

4.3.2 Smaller cystic echinococcosis control schemes

In several smaller hydatid control programmes or schemes undertaken in settled rural sheep–raising communities, e.g., Sanpete County (Utah, USA), Powys County (mid-Wales, UK), La Rioja (Spain) and Hutubi County (China), there were good outcomes reported for sheep and dog infection data within 3–10 years of interventions starting (Andersen et al., 1981; Palmer et al., 1996; Jimenez et al., 2002; Carmena et al., 2008; Zhang et al., 2009b). In these four areas human CE appears not now to be an important public health problem. A key feature for success was well structured and motivated veterinary teams and compliant endemic communities that were encouraged to accept control measures by targeted information and health education. Also all these schemes included either 'Tasmania-style' dog testing field clinics with arecoline (Utah) or 'Chilean-style' regular supervised dog dosing with PZQ (mid-Wales, La Rioja, Hutubi).

4.3.3 Transfer from attack to consolidation phase

One problem for hydatid control, whether a larger or a smaller programme, is sustainability of the 'attack phase' and timing for conversion to a surveillance-based 'consolidation phase'. The costly attack phase should be under effective veterinary services with existing proven rural outreach and have reliable annual funding planned for a minimum of 5–10 years of dog-targeted measures (Gemmell and Schantz, 1997; Gemmell et al., 2001; Lembo et al., 2013). For example, funding for the attack phase in the mid-Wales programme was cut after 6 years and that effectively converted the campaign from an Option 5 'vertical' programme (i.e., frequent dosing with PZQ) to an Option 2 style 'horizontal' programme based on health promotion only. This probably led to the reemergence of echinococcosis in sheep and dogs within 5–10 years of ceasing interventions (Lloyd et al., 1998; Buishi et al., 2005b; Craig and Larrieu, 2006). A well-funded hydatid control programme in Sardinia failed, in large part, because of poor outreach and poor acceptance by sheep farmers (Conchedda et al., 2002).

Successful transformation from the attack phase to a less costly consolidation phase has required effective meat inspection and subsequent quarantine of premises/farms/ranches with infected livestock and also

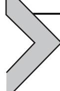
movement controls of livestock and dogs (Gemmell et al., 2001). This can be achieved in 10–15 years as occurred in Tasmania, Cyprus, Utah and Chile, but may take longer. Delayed transfer to a consolidation phase was the case in the New Zealand and Uruguay programmes where Honorary Commissions lacked the ability to undertake trace-back from abattoirs and were unable to enforce quarantine measures until they were restructured or replaced by the Ministry of Agriculture. Temporary or longer term reapplication of control measures, i.e., dog dosing, may be required during the consolidation phase as a result of trace-back of hydatid positive sheep at meat inspection, as occurred in Cyprus in the 1990s and in the Falkland Islands in 2000s (Lembo et al., 2013). Evidence of reemergence of hydatidosis in livestock in northern Tasmania in 2006 resulted in reactive local screening of farm dogs and high alert of authorities for potential increase in transmission (Jenkins et al., 2014). The important outcome of effective hydatid control is a significant reduction in incidence and prevalence of transmission in both livestock and dogs and in parallel fewer new human cases. However, interpretation of surveillance data needs care because a low prevalence situation, as a result of successful interventions, can lead to lower sensitivity and predictive values of surveillance measures, for example, copro-diagnostic tests in dogs and meat-inspection in lambs (Craig et al., 2015).

4.3.4 Control of cystic echinococcosis in semi-nomadic and poor pastoral communities

Hydatid control programmes have, perhaps, predictably fared less well when undertaken or attempted in underdeveloped regions characterized by transhumance pastoralism, semi-nomadism or nomadic lifestyles (Craig et al., 2007a; Lembo et al., 2013). For example, programmes undertaken in parts of East Africa, the Tibetan Plateau and in Central Asia. This may be due to many factors but include regions that are remote and harsh marginal zones, lack of roads and transport, poor general infrastructures, medically neglected illiterate populations, frequently hard-to-reach seasonally mobile populations of poor livestock keepers, lack of centralized livestock slaughter, and suspicious populations that are hard to engage (Macpherson, 1995; Zinsstag et al., 2006; Craig et al., 2007a). Planning and securing funding for control and appropriate surveillance from health or agriculture sectors are therefore difficult. Sustaining an effective attack phase (i.e., Options 3–6) that can reach >70% of owned dogs several times per year for several years is extremely challenging but has been at least

partially successful in Turkana nomad communities in northwest Kenya (Machpherson and Wachira, 1997) and in Tibetan semi-nomadic communities in northwest Sichuan Province (Heath et al., 2006).

In these kinds of remote endemic areas, it could be more effective to consider grouping together several zoonotic diseases (e.g., echinococcosis, brucellosis, rabies, anthrax and/or leishmaniasis) and even include nonzoonotic human diseases (e.g., TB, vaccine-preventable diseases, sexually transmitted diseases, gastrointestinal infections, nutritional deficiencies) in a 'One Health' approach of veterinary-medical cooperation. This could provide cost benefits and economies of scale and man power, more effective outreach and appropriate setting-specific multiintervention approaches (Schwabe, 1991; WHO, 2010a,b; Marcotty et al., 2013; Rabinowitz et al., 2013).



5. TARGETS AND TOOLS FOR CONTROL OF *ECHINOCOCCUS MULTILOCULARIS*

The life cycle (and therewith the zoonotic risk) of *E. granulosus* depends mainly on domestic animals, which are under direct control of the animal keepers. As for *E. granulosus*, domestic dogs can be an important or even the main source for human infections and should always be regarded as an important target for control measures against *E. multilocularis*. However, in contrast to *E. granulosus*, its life cycle is mainly maintained by wild intermediate and final hosts, which are much more difficult to manage than owned dogs and livestock. Even where domestic dogs are considered to be the main source for human infections, the cycle frequently is closely related to wild canids that contaminate rodent or other small mammal habitats with infective eggs. Therefore over large areas the main targets for the control of *E. multilocularis* life cycle are wild canids, mainly the adaptive and ubiquitous red fox (*Vulpes vulpes*) and abundant susceptible rodent species (mainly members of the family Cricetidae), which are frequently predated by final hosts.

Control and prevention measures for human AE can be taken at different levels (Hegglin and Deplazes, 2013). Hygiene-linked measures and frequent deworming of domestic dogs are important tools to reduce exposure to infective parasite eggs and can be pursued on an individual level. On an environmental level, measures to reduce the contamination with infective *E. multilocularis* eggs aim at the direct control of the parasite by deworming definitive hosts or at the control of the wildlife host populations. Population

control measures for the fox definitive host, mainly hunting, trapping and culling methods have been proposed in the past. However, also ecological changes (e.g., changes in agricultural methods or in the predator community) and their effect on host populations should be considered when interventions in the host populations are discussed (Hegglin and Deplazes, 2013). Japan and France have proven the feasibility to lower the infection pressure with *E. multilocularis* eggs by deworming red foxes on the basis of regular baiting campaigns.

5.1 Targetting fox populations for control of *Echinococcus multilocularis*

5.1.1 Culling fox populations

The substantial increase in prevalence rates and the spread of *E. multilocularis* to new regions observed in many European countries have been attributed to increasing population densities of red foxes after the eradication of rabies as a major mortality factor for this species (e.g., Schweiger et al., 2007). Therefore there is good reason to consider culling foxes as an effective measure to control *E. multilocularis*. Indeed hunting activities strongly affect wildlife populations. However, the effects of hunting and culling on wildlife can be very complex, and there is a broad debate on how such interventions are shaping red fox populations and if they really can contribute to lower the infection risk for human AE.

Heydon and Reynolds (2000) gave evidence that intensive culling under strict conditions can reduce fox population densities even in extended areas. Nevertheless, it is generally accepted that in most settings the regulation of fox populations is difficult to achieve on a larger scale (Baker et al., 2000). Hunting foxes is not as attractive as it was in the past, especially as fox fur prices are very low and therefore fox carcasses are usually disposed without making any use of the dead animals. Accordingly the population losses of regular hunting activities are easily buffered by a fox population, as the red fox has a high reproduction rate and any possible regulating effects of culling are hampered by compensatory mortality because the natural mortality in fox population is generally high. Furthermore, it is difficult to maintain a strong hunting pressure on a larger scale as fox hunting is time-consuming and requires substantial man power. Therefore, foxes can rapidly recruit and compensate for losses within a population or swiftly recolonize vacant territories (Newsome et al., 2014). There may also be ethical objections to fox hunting.

A strong compensatory density feedback was found to be acting through immigration, allowing red fox populations to resist high culling rates (Lieury et al., 2015). Furthermore, hunting can have strong impacts on the population dynamics (Minnie et al., 2016) and has to be considered in regard to disease transmission (Woodroffe, 2007). For instance, culling can increase the proportion of subadult foxes, which disperse over large distances (Harris, 1977; Harris and Trewhella, 1988). This could result in a higher spatial dynamic of parasite transmission and also boost the parasite biomass as subadult foxes can harbour higher worm burdens (Hofer et al., 2000; Morishima et al., 1999; Fischer et al., 2005). This assumption is supported by a recent French study. In a periurban area around Nancy, the proportion of immature foxes and also the prevalence of *E. multilocularis* (after an initial slight decrease) increased to significantly higher levels in areas with a high hunting pressure than compared to control areas (Comte et al., 2014).

The difficulty to control red fox populations is supported by observations from Australia where the introduced nonnative red fox population is treated as a pest species and thus much less protected by animal-welfare regulations in contrast to many other countries. However, although poisoning programmes are an accepted management method, control objectives are only partly achieved (Gentle et al., 2007). Hegglin et al. (2015) discussed possible behavioural effects of fox hunting that could be relevant for transmission of *E. multilocularis*. Hunting activity by humans can be regarded as a type of predation that not only has the direct effect of mortality but also results in behavioural responses of prey species to lower the 'predation' risk. In particular, this risk increases vigilance and decreases boldness. Shy wildlife is more restricted in its activity periods and its spatial behaviour and therefore has limited access to essential resources, which in turn could limit the population growth especially in and near urban settings (Kotler et al., 1994; Tambling et al., 2015). This hypothesis is supported by the fact that foxes — which are known nocturnal species — can quickly shift their activity pattern and become active during the daytime in reserves with no hunting activities (Servin et al., 1991). Cromsigt et al. (2013) discussed how such behavioural effects of hunting could be used for the management of wildlife by directly targeting the hunting strategies or the behavioural response of hunted species ('hunting for fear').

5.1.2 Praziquantel baits for fox populations

Baiting foxes has already been a very successful technique to control a zoonotic agent. In the 1960s the fight against the spread of rabies in Europe

started with strong efforts to control the fox population densities by extensive culling. However, control and finally eradication of rabies was only possible when oral vaccine baiting campaigns were initiated in the 1970s. In the late 1980s the first field trials were performed to assess the suitability of this successful technique also for the control of *E. multilocularis* by the delivery of deworming baits for foxes.

It was clear, however, from the beginning that the *E. multilocularis* cycle is much more resistant to such an intervention than rabies. Whereas rabies vaccinated foxes have lifelong protection from rabies virus infections, dewormed foxes (using PZQ baits) can be reinfected at any time after treatment as soon as they predate on infected intermediate hosts. A further challenge is longevity of the parasite eggs and the larval stages which are not affected by the anthelmintic treatment of foxes. The metacestode stages in the intermediate host and infective eggs in the environment can survive from several months to more than one year (Veit et al., 1995). This means that individual foxes have to be dewormed at regular intervals. The prepatency period for *E. multilocularis* is roughly 30 days. Therefore it is necessary to deworm individual foxes at monthly intervals if an intervention aims at completely disrupting the life cycle. Regarding these challenges the control of *E. multilocularis* by baiting red foxes is much costlier than control against rabies and it is unlikely to eradicate the parasite over large areas (Hegglin and Deplazes, 2013). However, different studies from Germany, Switzerland, Japan, Slovak Republic and France have proven the feasibility to lower infection pressure with *E.multilocularis* eggs by deworming red foxes based on regular baiting campaigns (see Table 5).

5.1.3 Fox population ecology

Fox densities can strongly be affected by infectious diseases like mange or rabies. In Great Britain and Sweden, sarcoptic mange was responsible for strong population declines of up to 95% (Baker et al., 2000; Soulsbury et al., 2007) and there is strong evidence that red fox populations were also heavily affected over larger parts of Europe by rabies during the epizootic periods in the 1960s (Chautan et al., 2000; Hegglin et al., 2015).

In many countries red fox densities have increased strongly after the elimination of rabies and reached densities that never have been recorded before. Also in regions where rabies never has been detected increases of fox populations have been recorded, e.g., Donaña National Park and the United Kingdom (Chautan et al., 2000). This long-term increase of many fox populations is considered to be related to the opportunistic feeding

Table 5 Experimental setting of different studies for the control of *Echinococcus multilocularis* by distributing Praziquantel baits for foxes

Positive samples after treatment (%)		**Low (<7%)**						**Medium (8–18%)**					**Failure**	
Study[a]		**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**J**	**E**
Treatment area (km^2)	Large (432–4568)	X	X					X						
	Medium (33–213)			X	X	X			X	X			X	
	Small (1–6)						X				X	X		X
Bait density ($baits/km^2$)	High (40–50)				X	X	X					X	X	
	Low (15–20)	X	X	X				X	X	X	X			X
Bait frequency (campaign/year)	Monthly (12)				X		X				X			X
	Not monthly (<10)	X	X	X		X		X	X	X		X	X	

The studies are grouped depending on the outcome of the experimental baiting measured by the investigation of fox intestines or copro-tests (low: significant decrease of parasite abundance end final portion of positive samples <7%; medium: significant decrease of parasite abundance end final portion of positive samples between 8 and 18%; failure: no significant effect on parasite abundance detected: (A) Schelling et al., 1997; (B) Tackmann et al., 2001; (C) Tsukada et al., 2002; (D) Koenig et al., 2008; (E) Comte et al., 2013; (F) Hegglin et al., 2003; (G) Romig et al., 2007; (H) Takahashi et al., 2013; (I) Inoue et al., 2007; (J) Antolova et al., 2006; (K) Hegglin and Deplazes, 2008.

[a]Analyses of parasite abundance by intestinal scrapping technique (A, B, D, G), sedimentation technique (I), necropsy (H), taenid eggs (C), copro-antigen ELISA (E, F, J, K).

behaviour of foxes as they can profit from increased agricultural productivity and a throwaway mentality in prosperous economies. In urbanized areas food resources for foxes are readily available. It has been shown that four average households in Zurich provide enough food (e.g., food waste, compost, garden fruits) to feed an adult fox (Contesse et al., 2004). Correspondingly, an Israeli study showed how red fox densities decreased after an experimental reduction of anthropogenic food resources (Bino et al., 2010). Thus the variation in the available food resources for foxes has to be considered as an important determinant to explain fox densities and therewith the transmission dynamic of *E. multilocularis*.

In many countries populations of large carnivores have decreased dramatically and became extinct over large areas during the 19th and 20th Centuries. It is likely that the red fox as a medium-sized predator profited also from this development as competition and intraguild predation is a strong driver of population dynamics in predator communities. It is known that larger canid species generally do not tolerate smaller canid species in their range. For example, it has been shown in North America that wolves

reduce coyote densities and coyotes in turn lower densities of grey, swift and red fox populations, by competition, agonistic behaviour and/or by direct predation (Berger and Conner, 2008; Fedriani et al., 2000). A similar pattern has also been shown in Australia where dingo abundance negatively correlates with fox abundance (Colman et al., 2014). In many regions of Europe, formerly extinct large predators like wolves and lynx are making comebacks as a consequence of strong protection and ongoing conservation programmes. This development possibly can affect the dynamic of red fox populations (Helldin et al., 2006; Ritchie and Johnson, 2009) and therewith also the dynamic of *E. multilocularis* transmission in those regions.

5.2 Targetting dogs for control of *Echinococcus multilocularis*

Dogs are an excellent host for *E. multilocularis* as evidenced from natural infections (Rausch et al., 1990; Craig et al., 1992; Budke et al., 2005c; Deplazes et al., 2011) and from experimental infections (e.g., Kapel et al., 2006). Dog ownership or contact in endemic areas appears to increase the risk of acquiring human AE (Kern et al., 2004; Wang et al., 2014). Therefore deworming dogs to reduce biomass of worms and environmental contamination is an important aspect of control especially to reduce the risk of human AE disease (Hegglin and Deplazes, 2013). Dogs have an important role in transmission of *E. multilocularis* in parts of Eurasia with epidemiological studies recording postmortem or arecoline purge prevalences of 10–19% in western China (Craig et al., 1992; Budke et al., 2005c), 18% in Kyrgyzstan (Ziadinov et al., 2008) and 5% in south Kazahkstan (Torgerson, 2013). The potential role of dogs in zoonotic risk for human AE in Europe is also gaining increased acknowledgement (Hegglin and Deplazes, 2013).

There are several studies, mainly in China and Central Asia that have demonstrated high prevalences of *Echinococcus multilocularis* in dogs (Budke et al., 2005a; Ziadinov et al., 2008). Furthermore, in regions with high prevalences of *E. multilocularis* in dogs there is frequently a high incidence of human AE (Craig et al., 2000; Tiaoying et al., 2005; Torgerson, 2013; Usubalieva et al., 2013; Wang et al., 2014, 2006a,b; Yang et al., 2006). It has been hypothesized that the close contact between dogs and humans may be an important factor resulting in the high incidence of human AE in such regions. Where dogs are infected, then routine treatment with PZQ is essential to prevent transmission to humans. As many of these areas are coendemic for *E. granulosus*, a dog treatment programme to control CE will contribute to ameliorating transmission of AE to humans. However, the

life expectancy of *E. multilocularis* in dogs is only about 90 days (Kapel et al., 2006) — somewhat shorter than the estimated life expectancy of *E. granulosus* of approximately 10–12 months (Torgerson and Heath, 2003).

This has a very important implications in populations of dogs with a similar prevalence of *E. granulosus* and *E. multilocularis* (for example, in Kyrgyzstan or the Tibetan plateau see Budke et al., 2005a; Ziadinov et al., 2008). There may be a much higher infection pressure and infection frequency of *E. multilocularis* to dogs compared to *E. granulosus*. So whilst treating dogs four times a year might be sufficient to reduce the transmission of *E. granulosus* it might have much less effect on *E. multilocularis* because of the probable more rapid reinfection rate. Therefore an increased frequency of treatment might be required. It also appears likely that in these communities *E. multilocularis* has developed an anthropogenic cycle between dogs and small rodents. This would help facilitate the control of AE as dosing of dogs would then reduce infections in rodents with a subsequent negative feedback to dogs and long-term reduction in infection pressure (Moss et al., 2013). In contrast if it were purely a spill over from a fox–rodent cycle, the infection rates in rodents would remain unaffected by treatment of dogs as they would be continuously be infected from foxes. In such a scenario the routine treatment of dogs would be seen as a permanent measure to prevent human infection (Rausch et al., 1990).

Where dogs are an important definitive host of *E. multilocularis,* then many of the schemes to control dog populations described for the control of *E. granulosus* would be applicable to prevent AE transmission. High prevalences in dogs are often found in communities, such as the Tibetan plateau or rural Kyrgyzstan, where there is widespread poverty. A consequence of this is that dogs may not receive enough food from their owners so are forced to scavenge or hunt small mammals such as rodents. Ziadinov et al. (2008), for example, found that free-roaming dogs in central Kyrgyzstan were more likely to be infected with *E. multilocularis*.

5.2.1 Praziquantel dosing of dogs for Echinococcus multilocularis

Experimental infections in dogs can be terminated using PZQ at 5 mg/kg (Eckert et al., 2001). The prepatent period of *E. multilocularis* in experimentally infected dogs was around 30 days (Kapel et al., 2006) and therefore a monthly (4 weekly) dosing frequency (rather than six weekly as recommended for *E. granulosus*) would be justified from a theoretical transmission viewpoint. In areas where human AE and human CE are coendemic (e.g., Kyrgyzstan, northwest Sichuan), then regular dosing of owned dogs

with PZQ at a frequency of 4–8 times per year, as routinely carried out for control of *E. granulosus* (see Section 2.2.2), will also have an impact on *E. multilocularis* and reduce the risk human AE as well as CE. However, because of the shorter prepatent period for *E. multilocularis* in dogs (i.e., 30 days vs. 42 days for *E. granulosus* s.l) the potential for reinfection of dogs with *E. multilocularis* (if they predate/scavenge small mammals) would be greater than for *E. granulosus*.

A reinfection study that followed a cohort of 276 owned dogs in Tibetan communities of Shiqu County (Sichuan) after a single treatment with PZQ indicated a baseline copro-PCR prevalence for *E. multilocularis* of 11.2% and a reinfection prevalence of 2.9% after only 2 months posttreatment (Moss et al., 2013). In such regions monthly dog dosing with PZQ would be the gold-standard to reduce or eliminate viable canine infections with *E. multilocularis*, but seasonal factors and semi-nomadic movements make that extremely difficult to achieve. Very few *Echinococcus* control programmes could effectively undertake monthly dog dosing for sustained periods (>3 years) even if they have good resources. Nevertheless, this has been recommended for western China because both CE and AE are coendemic in many regions (Craig, 2004; Li et al., 2010; Huang et al., 2008; WHO, 2011), but this is very unlikely to be achievable in all areas (van Kesteren et al., 2015).

An intensive dog-dosing scheme using PZQ (5 mg/kg) at monthly intervals for 10 years was introduced into an Inuit community on St Lawrence Island (Alaska) through the 1980s to reduce the village-level transmission of *E. multilocularis* and eliminate human AE as a public health problem (Rausch et al., 1990). The impact of dog-dosing intervention was measured by monitoring prevalence of AE infection in commensal vole populations of *Microtus oeconomus*, which decreased from 29% to 1–5% after 10 years, despite the continued transmission between voles and arctic foxes outside the village (Rausch et al., 1990).

5.2.2 Dog population management and control of alveolar echinococcosis

The same considerations and criteria apply here as described in Section 2.2.3. In some AE endemic regions in Asia, stray dog, unowned dogs and/or community-owned free-roaming dogs, as well as free-roaming owned dogs, can potentially contribute to the zoonotic risk and even transmission of *E. multilocularis* (Budke et al., 2005a; Vaniscotte et al., 2011; Moss et al., 2013; van Kesteren et al., 2013). In Tibetan communities in Ganze Prefecture

(Sichuan, China) presence of a Buddhist temple in or near a village often increased stray dog or community-dog populations because monks usually feed dogs. Culling dog numbers or impounding animals in such communities is either not accepted or not sustainable (Wang et al., 2014). The use of PZQ baits to dose stray dogs may be a useful approach, if affordable.

5.3 Small mammal populations and control of *Echinococcus multilocularis*

Rodents and other ground-foraging small mammals are an important component of the food web and can provide ecosystem services such as soil aeration and fertilization (Jacob et al., 2014; Martin, 2003). However, some rodent species can cause substantial economic losses due to their high reproductive rate and the high densities they can reach. For example, in Europe two fossorial rodent species, the common vole (*Microtus arvalis*) which, together with *Arvicola scherman*, is considered as the most important intermediate host for *E. multilocularis* over a large part of Europe can begin reproducing at only two weeks of age (three week gestation period) with an average five to six pups per litter and 4.5 litters per breeding season thereby reaching population densities of more than 2000 individuals per ha (Jacob et al., 2014). Due to regular population outbreaks they can cause substantial economic damage to grasslands, e.g., in Poland, common voles accounted for a financial loss of about 3.5% of farmers' income (Truszkowski, 1982).

Despite the strong interest of the agriculture sector to prevent rodent induced damage, there are no simple measures to control such pest species sustainably in the long-term. Although rodenticides can effectively reduce rodent populations over large areas (Tobin and Fall, 2004), due to the high reproduction rates many rodent species are resilient to such interventions (Jacob et al., 2014). Furthermore, such interventions can heavily affect the environment by secondary poisoning of predators such as mustelids, canids and raptors (e.g., Giraudoux et al., 2006b) and may also poison domestic dogs (Giraudoux et al., 2013a). Furthermore, it has to be considered that foxes may have certain prey preferences. For example, *Microtus arvalis* is a very attractive prey to red foxes in Europe (Macdonald, 1977). Therefore certain vole species can be predated frequently (and therewith be important for the parasite transmission), even when they occur at low densities (Hegglin et al., 2007). Correspondingly, only minor increases in the abundance of *Arvicola scherman* and *M. arvalis* host species were linked with a strong increase in the infection levels in foxes (Raoul et al., 2010, 2015).This means only when intermediate host populations, of such

preferred prey species, can be reduced to a very low level can a substantial decrease of prevalence rates in foxes be expected.

In southern Switzerland, where seven arvicolid species occur, it has been shown how the distribution of *E. multilocularis* is closely linked to the distribution of *M. arvalis* but not to the other six arvicolid species that occur in the region (Guerra et al., 2014). A Japanese study demonstrated how changing vole densities affected the prevalence rates in red foxes (Saitoh and Takahashi, 1998). Thereby, it has to be considered that agricultural practices and landscape management are important determinants for rodent communities and thus can shape the transmission dynamics of *E. multilocularis* (Giraudoux et al., 2002; Viel et al., 1999). Thus changes in land management practices could have considerable influence on the incidence rates of AE in humans by influencing rodent communities (Giraudoux et al., 2006a; Viel et al., 1999; Wang et al., 2006b). For example, it has been shown in the Massif Central, France, that the risk for outbreaks of *A. scherman* populations is high in regions where the proportion of permanent grassland exceeds 90% and low in regions where this proportion is <80% (Fichet-Calvet et al., 2000). It is, therefore, worth considering how agricultural practices and landscape management sustain high densities of known or potential intermediate hosts (Giraudoux et al., 1997). Altering the habitat and reducing its potential for maintaining populations of relevant small mammal intermediate host species near human settlements could play an important role in the framework of integrated control programmes (Hegglin and Deplazes, 2013; Giraudoux et al., 2013b).

5.4 Modelling the transmission of *Echinococcus multilocularis*

There have been a number of mathematical models developed to describe the transmission of *E. multilocularis*. These include relatively simple extensions of the models developed by Roberts for *E. granulosus* (Roberts, 1994). This model has been used to consider the infection pressure to dogs in China (Budke et al., 2005b) and to describe the force of infection to foxes in Switzerland (Lewis et al., 2014). In this case the models have a simple 'black box' approach that only examines the infection pressure to the definitive host and some aspect of parasite–host ecology. It does not incorporate any analysis of the population dynamics of rodents.

Despite this relative simplicity, the models can estimate the likely frequency of infection and any seasonal variations in such force of infection. Thus it should be possible to use the results of such a model to target fox baiting in the most

cost-effective manner (Lewis et al., 2014). Thus baits could be distributed more frequently in seasons when the force of infection is at its highest and less frequently at other times of year. This would likely be both more effective and more cost-effective than simply distributing baits at a fixed frequency. Other models have demonstrated that once treatment has successfully reduced fox prevalence, treatment of the definitive host needs to continue as otherwise the parasite is likely to reemerge from a remaining persistence of the larval stage in intermediate hosts (Takumi and Van der Giessen, 2005). This has been confirmed by field observations in Germany and Switzerland where there was a rapid rebound in fox prevalence following cessation of baiting campaigns (Hegglin and Deplazes, 2008; Romig et al., 2007).

More complex models have also incorporated seasonal dynamics of rodent populations which also helps to clarify the dynamics of seasonal transmission (Ishikawa et al., 2003). These models also can be used to estimate the basic reproductive number or R_0 and possibly what type of interventions could drive R_0 below 1, which is required to control or eliminate the parasite. However, the force on infection rather than R_0 could be a more useful parameter to estimate from the point of view of control (Lewis et al., 2014).

Another important application of a transmission model has been to estimate the risk of introducing the parasite into a previously nonendemic area by dogs. The United Kingdom, Ireland, Finland and Malta currently have derogations from EU law that allows them to obligate the praziquantel treatment of dogs before the dog is allowed into the country. Torgerson and Craig (2009) used this model to estimate the probability of a dog becoming infected whilst resident or visiting an endemic country such as Germany. Based on the number of dogs entering the United Kingdom and this probability of infection it was possible to conclude that it was virtually inevitable that *E. multilocularis* would be introduced into the United Kingdom if the obligation to treat with PZQ immediately prior to importation was abandoned.

More complex spatial models have modelled the distribution of *E. multilocularis* and could be used to predict the likely future expansion of endemic areas (Staubach et al., 2011; Takumi et al., 2008). Spatial models have also been used to describe and predict the endemic areas of *E. multilocularis* and possible risk of human disease. These have been developed in Germany (Berke, 2001; Berke et al., 2002; Staubach et al., 2011), Belgium (Vervaeke et al., 2006), France (Pleydell et al., 2004) the Netherlands (Takumi et al., 2012) and China (Danson et al., 2003; Graham et al., 2005, 2004; Giraudoux et al., 2013a).

5.5 Health education and prevention of human alveolar echinococcosis

Educational efforts to prevent AE will depend on the major transmission routes to humans. Where dogs are the main source of human infection, then similar efforts to that of CE with an emphasis on dogs is warranted. Here the importance of prevention of dogs scavenging rodents, regular anthelmintic treatment of dogs and routine hygienic precautions taken when there is contact with dogs. As with the case of *E. granulosus*, food is also a possible vehicle of transmission (Torgerson et al., 2015) and the same recommendations for ensuring kitchen gardens are secure from dog (or fox) faeces and the safe preparation of fresh produce can be made. In Europe, it has been shown that the infection risk is clearly linked to a rural life style with working in agriculture or growing own vegetables as important risk factors (Kreidl et al., 1998; Kern et al., 2004; Piarroux et al., 2013). It was further suggested that wild berries, contaminated with fox faeces might also be responsible for transmission to humans. However, there is no epidemiological evidence that this is a major transmission pathway (Kreidl et al., 1998; Kern et al., 2004; Piarroux et al., 2013). Nevertheless, in a recent Polish study, *E. multilocularis* DNA has been reported from 23% of environmental samples including fruits and vegetables collected from forests, but also from plantations and kitchen gardens in Poland (Lass et al., 2015). However, considering the generally low prevalence rates found in wild rodents such a high degree of environmental contamination seems rather unlikely. But generally in Europe such recommendations will depend on the knowledge with regard to *E. multilocularis*, which seem to vary across the European endemic region. For example, fewer people had heard of *E. multilocularis* in the Czech Republic (14%) and France (18%) compared to Germany (63%) and Switzerland (70%) (Hegglin et al., 2008).

6. SURVEILLANCE FOR *ECHINOCOCCUS MULTILOCULARIS*

6.1 Surveillance of alveolar echinococcosis in humans

In Europe where human AE is a rare disease, hospital records and specific registers are important for epidemiological and surveillance data and are reasonably reliable when based on histo-pathological and/or molecular DNA confirmation (Kern et al., 2003; Vuitton et al., 2003, 2015; Said-Ali et al., 2013). In underdeveloped resource-poor endemic

regions, for example, in Central Asia and western China, hospital records may be of some value (e.g., Raimkylov et al., 2015), but often do not clearly differentiate AE and CE cases, may misdiagnose AE disease, and pathological details may not be recorded effectively. Furthermore, hospital records are usually more reliable for human CE and may not even closely reflect the burden of human AE disease in the community. This is because human AE usually has a longer asymptomatic period and is also more likely to cause nonspecific symptoms. For example, hospital records examined in south Ningxia Hui Autonomous Region (northwest China) confirmed that 96% of hospital cases were due to CE; however, community mass screening by ultrasound showed that 56% of hepatic cases detected in the surrounding rural communities were actually due to AE and only 44% detected were confirmed as CE (Yang et al., 2006).

6.1.1 Active mass screening for human alveolar echinococcosis

Serological diagnosis for human AE is relatively sensitive and specific for antibody detection in advanced cases using either native antigens or recombinants (Sako et al., 2010) and has been applied as a primary mass screening tool for AE in parts of Europe, northwest China, northern Japan and Alaska, where seropositives were then clinically followed up (Gottstein et al., 1985; Craig et al., 1992; Bresson-Hadni et al., 1994; Ito et al., 2003a). Test sensitivity should be maximized to increase the likelihood of case detection because of the high fatality rate of untreated AE cases (Bartholomot et al., 2002). A seropositive test on its own does not confirm an AE diagnosis and requires an image-based follow-up investigation, e.g., by ultrasound, CT scan, and/or MRI (Brunetti et al., 2010).

Mass screening of humans in resource-poor AE endemic zones in China has been undertaken successfully using portable ultrasound scanners (Craig et al., 1992; Macpherson et al., 2003; Tiaoying et al., 2005; Yang et al., 2006). Furthermore, ultrasound data have been used to provide baseline data and to inform the progress of CE/AE control programmes in China (Li et al., 2010; WHO, 2011). One problem encountered for AE diagnosis during community ultrasound screening in highly endemic (especially underdeveloped) areas, is the occurrence of small hepatic lesions (0.5–2 cm) of unknown aetiology that could be early AE disease, abortive AE lesions, or due to other causes (e.g., haemangiomas, TB, ascariasis). Serological confirmation may be useful for these query cases (Bartholomot et al., 2002; Yang et al., 2007).

6.2 Surveillance of *Echinococcus multilocularis* in foxes

In recent years, new highly sensitive and specific diagnostic strategies have been developed and knowledge about the spatial distribution is increasing year by year. However, the distribution of the parasite's range is dynamic (Davidson et al., 2012), and there is a need for defining minimal requirements and harmonised approaches for assessing the epidemiological situation and generate comparable results over different countries (Conraths and Deplazes, 2015).

6.2.1 Necropsy

The collection and dissection of foxes is still the most widely used approach to monitor occurrence and the only method allowing estimation of the abundance of *E. multilocularis*. Although applied laboratory techniques (see below) have a high specificity and a sufficient sensitivity for most purposes, they are very laborious and depend on access to fresh fox carcasses and safety requirements (Conraths and Deplazes, 2015). The investigations rely in most cases on hunted foxes during the regular hunting season, or which were found as road kills. This sampling has to be critically assessed as hunting activities are seasonally restricted (mainly winter) and shot foxes do not reflect a random sample (Tryjanowski et al., 2009). Furthermore, hunting interventions affect the fox population dynamic, age structure and the spatial behaviour of the fox population under study which in turn could affect the transmission dynamics of the parasite (Conraths and Deplazes, 2015; Hegglin et al., 2015).

The SCT in several modifications and the less laborious but also less sensitive intestinal scraping technique are the two standard techniques to isolate and identify *E. multilocularis* from the intestines of final hosts (Conraths and Deplazes, 2015). These methods rely on the morphological identifications of *E. multilocularis* and are herewith highly specific (unless in areas where coinfection with *Echinococcus granulosus* are likely). They allow the determination of the development stages (premature, mature and gravid) and to perform quantitative analysis of the parasite burden. The SCT is intended to determine the total biomass of the parasite and has an estimated sensitivity of 83% as determined by a recent comparative study with a highly specific copro-PCR (Wahlstrom et al., 2016).

6.2.2 Serology for Echinococcus multilocularis in foxes

Crude parasite antigens or affinity-purified Em2 antigen in ELISA have not been considered as suitable for serological screening mainly due to the

persistence of antibodies after elimination of the cestodes and the poor correlation between the presence of specific antibodies in the serum and worms in the intestine (Conraths and Deplazes, 2015; Craig et al., 2003).

6.2.3 Copro-tests for Echinococcus multilocularis

The detection of *E. multilocularis* infection in final host populations by coprological tests has several advantages. The sampling does not rely on dead animals and therefore does not affect the population under study. Furthermore, the sampling of faeces has also no seasonal restriction as it is the case for hunting that has to follow the regulations of the local game law and respect closed periods. In addition some coprological methods are rather efficient, e.g., coproantigen ELISA, and are therefore very suitable for monitoring studies over large areas (Sakai et al., 1998; Deplazes et al., 1999). On the other hand, studies based on fox scat samples collected in the field are confronted with several challenges. These include difficulty to assess to which extent different vegetation types and weather conditions affect the detection rate for faeces. Furthermore, the identification of carnivore faeces based on morphological features can sometimes be difficult when no molecular techniques are used to confirm proper identification. Most importantly the sampling of faeces in the field is not suited for determining prevalence rates, as it is difficult to exclude that several samples from one individual have been collected unless genetic analyses, allow the determination of individuals.

Classical routine diagnostic methods to concentrate proglottids and worm eggs from faeces for microscopical detection lack sensitivity and specificity. The morphological differentiation between *E. multilocularis* and other taeniid eggs is not possible. However, an efficient technique to concentrate taeniid eggs with a combination of sequential sieving and flotation in zinc chloride solution (F/Si-method) (Mathis et al., 1996) followed by PCR analyses is a widely used method to identify patent *E. multilocularis* infections. In an experimental study with foxes, the sensitivities for this method were 100% for high patent (30–70 dpi) and 80% for low patent infections (71–90 dpi) (Al Sabi' et al., 2007).

Another approach is direct copro-DNA isolation and amplification; several PCR approaches have been validated and used in epidemiological studies (Conraths and Deplazes, 2015). The most sensitive (81% and 96% for foxes with less and more than 100 worms, respectively) and the costliest method was recently developed for extended studies in a low endemic *E. multilocularis* area in Sweden (Isaksson et al., 2015). This semi-automated

magnetic capture probe-based DNA extraction and real-time PCR method (MC-PCR) proved to be similar in sensitivity and specificity as the SCT (Wahlstrohm et al., 2016).

Sandwich ELISA has been proven to be a very efficient way to detect *E. multilocularis* coproantigens in field samples of fox faeces (several tests have been validated and are summarized in Conraths and Deplazes (2015). With this approach 500–800 field samples can be analyzed by one trained person per five working days, which is roughly 5 to 10 times less time-consuming than most PCR techniques (Conraths and Deplazes, 2015). Another advantage is that prepatent infections can also be detected. Depending on the test, sensitivities of 80–87% (compared to SCT) and specificities of 70–95% have been recorded. A recent study based on a latent class analysis revealed a sensitivity of only 55% in dogs (Hartnack et al., 2013). Considering the limited specificity this technique can be used to monitor low endemic areas only when ELISA positive samples can be confirmed with PCR analyses.

6.3 Surveillance of *Echinococcus multilocularis* in dogs

The tools and approaches available for detection and surveillance of *E. multilocularis* in dogs are essentially the same as described for infection in foxes (Section 6.2), i.e., necropsy, coproantigen ELISA and coproPCR, but also includes arecoline purgation as described for detection of *E. granulosus* in dogs (see Section 3.2).

The small average size of *E. multilocularis* tapeworms (2–3 mm), however, presents potential difficulties for necropsy and purge examination, especially in the field, and when worm burdens are low. At necropsy and examination of the dog small intestine (preferably after deep freezing at −80°C for 5 days) the sedimentation and counting technique is a gold-standard for sensitivity and specificity (close to 100%), reported to detect single worm burdens in foxes (Hofer et al., 2000). In AE/CE coendemic areas, it is important to identify samples of adult worms recovered from dogs by morphological or PCR methods (Budke et al., 2005c; Craig et al., 2015). When arecoline purgation is used on dogs then washed purges need to be carefully examined (preferably after boiling or formalin fixation or after bagging and freezing at −80°C) on a black background with a magnifying glass or low power microscopy. *E. multilocularis* infections, including mixed infections with *E. granulosus* s.l, have been detected after arecoline testing, in owned dogs in Tibetan (Budke et al., 2005c) and

Kyrgyz (Ziadinov et al., 2008) pastoral communities that were coendemic for human AE and CE.

Coproantigen detection is a useful primary screening test for *Echinococcus* spp. in dogs (and cats), but is not able to reliably differentiate *E. multilocularis* and *E. granulosus* s.l infections (Mathis and Deplazes, 2006; Allan and Craig, 2006; Huang et al., 2013). Secondary screening using a copro-PCR to amplify species-specific DNA is currently the only laboratory test method to confirm *E. multilocularis* infection in dogs (or foxes) (Dinkel et al., 2011; Boufana et al., 2013; Wahlströhm et al., 2016). This makes mass screening of dogs more difficult, expensive and time-consuming, but can provide useful surveillance data for intervention programmes and epidemiological studies, especially in coendemic areas (Ziadinov et al., 2008; Moss et al., 2013). DNA tests also have the potential to confirm whether dogs and foxes in a transmission zone are infected with the same haplotype of *E. multilocularis*, for example, as described in south Kyrgyzstan (Afonso et al., 2015).

6.4 Surveillance in small mammals

Studies on the distribution and abundance of *E. multilocularis* are generally based on investigations on final hosts as prevalence rates combined with host density estimates can be used to directly assess the environmental contamination with infective *E. multilocularis* eggs. Furthermore, final hosts roam over much larger areas than small rodents and better reflect the parasite population dynamics on a larger scale. However, it is crucial to determine which species act as intermediate hosts to understand the transmission pathways in a given region. With this approach different transmission studies have been described in China (Giraudoux et al., 2013b).

It, therefore, has to be considered that *E. multilocularis* infections in rodents frequently are very heterogeneously distributed over space and time (Liccioli et al., 2014; Burlet et al., 2011). This makes it difficult to get representative samples to assess comparable prevalence rates across different wild rodent species and over larger areas. Thereby, it is important to note that the predation of final hosts on different intermediate host species is a very selective process. Red foxes show preferences for certain prey species and *Microtus* species appear to be more attractive than other arvicolid species, and arvicolid species in general are more attractive than murid species (Macdonald, 1977; Green, 2002). Furthermore, the predation rates on different species can depend to a large extent on the density of a specific rodent and of alternative prey species (Raoul et al., 2015). Therefore, it is

important to assess not only the prevalence rates of different rodents species but also to which extent these species are predated by the final hosts (Hegglin et al., 2007).

Investigations on small mammals rely on the dissection of trapped animals and the careful examination of the liver for suspicious lesions. Fertile infections can be identified by microscopical analyses of the suspected metacestode tissue for protoscoleces. Whenever possibly the number of protoscoleces should be estimated to get data on the parasite fertility in different intermediate host species. Visually unidentifiable lesions should be investigated by a PCR specific for *E. multilocularis*. As the age structure of rodent populations usually vary over seasons and years and the prevalence rates increase with age, it is recommended to use age indicators for the dissected rodents (eye lens weight Burlet et al., 2011).

When the situation is very unclear, it is advisable to make exploratory studies where rodent trapping is based on assumptions about the predation on intermediate hosts, where predation is expected and final hosts defecate. Such defined places are supposed to be hot spots and can be starting points to understand the role of different rodent species in a given area.

In some circumstances the trapping of rodents can also be used to document changes in environmental egg contamination. For example, in Zurich a control study investigated the prevalence rates in *A. scherman* in baited and unbaited areas. In this study a lower prevalence in baited studies documented the lower contamination and the lower reinfection level in baited areas. Furthermore, only the abundance of *A. scherman* could be used as an indicator of higher human AE infection pressure (Viel et al., 1999).

7. CRITICAL APPRAISAL OF ALVEOLAR ECHINOCOCCOSIS CONTROL PROGRAMMES

7.1 Island programmes for alveolar echinococcosis control

7.1.1 Reuben Island (Japan)

An early example of the successful control of *E. multilocularis* has been reported from Rebun Island in Japan (Ito et al., 2003a). In 1937 a first case of human AE was diagnosed on this small island, which comprises an area of not more than 83 km^2. In the following decades human AE became highly prevalent and until 1964 a total of 111 patients had been diagnosed for this previously unknown disease on the island. This sudden

occurrence of human AE was linked with the introduction of 12 pairs of red foxes, which were imported between 1924 and 1926 for the control of voles and the production of fur (Takahashi et al., 2005). It is reported that poachers were successful in completely eradicating foxes on this island after 1935. In the early 1950s, more than 2000 foxes and 3000 dogs were killed (Eckert et al., 2001) and in the framework of a control programme dogs and cats were captured and autopsied until 1970 (Minagawa, 1999). These efforts proved to be very successful. After 1964 the number of newly diagnosed AE patients dropped sharply and since 1994 no new records of human AE have been registered giving evidence that it was possible to eradicate the parasite completely from the Island (Ito et al., 2003a).

7.1.2 Hokkaido (Japan)

In 1965 another AE endemic area was detected in the Nemuro district, in the eastern part of Hokkaido main island (Takahashi et al., 2005). A total of 148 human AE cases have been recorded between 1965 and 1997 whereby the prevalence rates in foxes strongly increased between 1985 and 1999 (Ito et al., 2003a). The life cycle depends to a large extent on red foxes and their predation on *Clethrionomys* species mainly *Clethrionomys rufocanus* (Takahashi et al., 2005) which lives in the undergrowth of forests and bushland.

Different baiting studies in Hokkaido have demonstrated lower environmental contamination with *E. multilocularis* eggs following the delivery of anthelmintic baits for foxes. PZQ baits have been placed over an area of 90 km^2 near fox dens in monthly intervals during a 13 month period (Tsukada et al., 2002). Taeniid egg detection in fox faeces decreased from 27% to 6% and the prevalence in *C. rufocanus* born after the onset of the baiting campaigns was significantly lower in the baited areas than compared to the nonbaited areas (1.7 vs. 13.5%). A second control study has been conducted in the Nemuro peninsula over an area of 135 km^2 where commercially available PZQ baits were distributed along roads (bait density: 15 baits/km^2) and additionally around fox dens. The prevalence in foxes decreased from 49% to 16% with 27 baiting campaigns during 63 months, whereas it remained stable in a control area of 27 km^2 (Takahashi et al., 2013). Also the distribution of PZQ baits in a highly epizootic suburban area of Otaru proved to be successful in lowering the prevalence in foxes during a period of 43 months with 14 treatments (20 baits/km^2) from 58% to 11% (Inoue et al., 2007).

7.1.3 St. Lawrence Island (Alaska)

A successful control programme was implemented on St. Lawrence Island in Alaska in the late 1970s, where domestic dogs preying on tundra voles (*M. oeconomus*) in villages were frequently infected with *E. multilocularis*. In this area the life cycle depends on the predation by the arctic fox (*Vulpes alopex*) on these voles (Rausch et al., 1990). Before the treatment started the prevalence rates in *M. oeconomus* ranged from 22 to 35% (mean 29%). After 2 years of a monthly delivery of PZQ to owned dogs, the prevalence rates in *M. oeconomus* dropped to a relatively stable value of 5%, thus not only demonstrating that the treatment was effective in establishing a lower reinfection pressure on the dog population but also that the rodents around the villages got infected mainly by parasite eggs excreted by domestic dogs and not arctic foxes (Rausch et al., 1990).

7.2 Continental programmes for alveolar echinococcosis control

7.2.1 Germany

In the late 1980s the first field experiment was initiated to investigate the feasibility for control of *E. multilocularis* by the delivery of anthelmintic baits to foxes in south-western Germany (Schelling et al., 1997). This study over a baiting are of 566 km^2 revealed a decrease of *E. multilocularis* prevalence in foxes from 32% (95% CI; 16—52%) to 4% (2—7%) after six baiting campaigns within 14 months. A follow-up study confirmed the success of the bait delivery. In baited areas the baseline prevalence of 64% (59—69%) decreased to 15% (95% CI; 10—21%) whereas the prevalence rates in foxes in the control area remained stable (Romig et al., 2007). After reducing the baiting intervals to 6 months, the prevalence increased during 15—21 months to 31—41% (95% CI) and finally to 49—61% in 13—18 months after the last bait distribution (Romig et al., 2007).

A field study using PZQ baits in north-eastern Germany was also successful to lower prevalence rates from 16—27% to 2—6% in an endemic foci and from 4 — 7% to 0—1% in an area of low endemicity by baiting at six-week intervals for one year, followed by a second year with three-month intervals (Tackmann et al., 2001). Also a fourth control study in the south-east of Germany was successful and reduced the fox prevalence rate from 35% (95% CI; 22—50%) to a very low prevalence of 1% (0—4%) (Koenig et al., 2008). The authors of this study conclude that the strong decrease was linked to a high baiting frequency (monthly), high bait

density (50/km^2) and the good coverage of the baiting area with the inclusion of densely populated areas.

7.2.2 Switzerland

In Switzerland several consecutive anthelmintic baiting studies have been performed in the city of Zurich. Whereas the baiting studies in Germany aimed to control the parasite in extended areas over areas of 213 km^2 (Schelling et al., 1997) up to 4568 km^2 (Tackmann et al., 2001), the areas in Switzerland comprised only a set of small experimental plots (areas of 1–6 km^2), which were situated in the urban periphery of the city of Zurich (Hegglin and Deplazes, 2008; Hegglin et al., 2003). In this transition zone between rural and urban areas a high population density of foxes was sustained by the rich anthropogenic food resources and at the same time foxes had access to suitable intermediate host species, like *A. scherman* and *M. arvalis* and therefore were frequently infected with *E. multilocularis* (Hegglin et al., 2007; Stieger et al., 2002). Therefore these highly populated urban areas are considered to be especially relevant for the potential transmission of human AE (Deplazes et al., 2004). Surprisingly the delivery of baits on a monthly basis was very effective even on plots of only 1 km^2 (decrease of coproantigen positive fox faeces from 39% to 6%) and was even shown to be successful at lowering the reinfection pressure to rodent intermediate hosts in which the AE prevalence significantly dropped from 7.3% to 2.1% (Hegglin et al., 2003).

Furthermore it has been shown in Zurich that over an area of only 6 km^2 which was baited during a 3.5-year-period at monthly intervals, the contamination was still very low for 3 years after all bait delivery ended (Hegglin and Deplazes, 2008). The feasibility of successful baiting was attributed to the fact that resident foxes in the urban area of Zurich have very small home ranges, i.e., mean home range sizes: females 29 ha, males 31 ha (Gloor, 2002) and therefore the spatial dynamic is far less pronounced than in areas where foxes have larger home ranges and spatial organization is more disturbed by stronger hunting activities (Hegglin et al., 2015).

7.2.3 France

So far experimental fox baiting campaigns to control *E. multilocularis* in France have also concentrated on urban areas. During 32 months, 14 baiting campaigns were performed in the two medium-sized cities of Annemasse and Pontarlier (Comte et al., 2013). The treated areas comprised in each city an area of 33 km^2 and 40 baits had been distributed per km^2 and baiting

campaigns. The study achieved contrasting results between the two cities. Whereas the contamination with *E. multilocularis* positive fox faeces decreased significantly from 13.3% to 2.2% in Annemasse, no significant change was detected in Pontarlier (i.e., 9.1%) where the contamination of the treated area followed the temporal trend observed in the control area. It was supposed that the greater resilience of the parasite's life cycle in this city was related to a strong pressure of recontamination from outside the treated area. These contrasting results give evidence that the intensity of the control efforts have to be adjusted to regional needs.

7.2.4 Eastern Europe

During a field study in the Slovak Republic two areas of 2 km^2 each were treated during 9 months with a monthly delivery of 20 PZQ baits per km^2 (Antolova et al., 2006). Similar to the French studies, two contrasting results were achieved in the two baiting areas. Whereas in one area the portion of *E. multilocularis* positive fox faeces dropped significantly from roughly 38% to 8% (Antolova et al., 2006). However, no significant change was observed in two control areas and in the second baiting area where it remained stable on a level between roughly 40–60%. The failure in the second baiting area was attributed to the high density of wild boar in this area, which most likely consumed a substantial amount of the distributed baits (Antolova et al., 2006).

7.2.5 Western China

The highest global burden of human AE disease occurs in west China where prevalences ranged from <1% to >10% in upland agricultural or high-altitude pastoral communities (Craig, 2006; Li et al., 2010; Torgerson et al., 2010). The highest numbers of AE cases occur in Tibetan pastoral communities above 3500m altitude in Sichuan, Qinghai and Tibet Autonomous Region (Giraudoux et al., 2013a), but significant numbers also in lower altitude (<2500m) Han and Hui farming communities in south Gansu and south Ningxia (Craig et al., 1992; Yang et al., 2007). The China National Echinococcosis Control Programme consequently is required to consider control of not only CE but also AE in these areas many of which are coendemic. Dogs are known to be infected in all these zones and thus regular anthelmintic dosing would be expected to reduce the prevalence of both *E. granulosus* and *E. multilocularis* (Wang et al., 2014). In Shiqu County in northwest Sichuan Province a natural reinfection study in owned dogs after a single PZQ dose, showed that at baseline using copro-PCR

11.2% of dogs had *E. multilocularis* DNA positive faeces and after two months posttreatment 2.9% were copro-PCR positive, i.e., reinfected; in comparison *E. granulosus* prevalence was 3.6% at baseline and 0.5% after 2 months (Moss et al., 2013).

The transmission ecology of *E. multilocularis* in western China and Central Asia is complex with three species of fox host including the red fox, Tibetan fox (*Vulpes ferrilata*) and corsac fox (*Vulpes corsac*) and a large number of small mammal families and species potentially able to transmit *E. multilocularis*. Understanding small mammal host ecology and how landscape can affect their population distribution and densities have important practical applications. Spatially explicit models were constructed to investigate the epidemiology of human AE in south Gansu and Ningxia (Danson et al., 2003, 2004), and these were later refined to enable predictive approaches to identify communities at particular risk of AE at local and regional landscape levels (Giraudoux et al., 2013a).

7.3 Reasons for success and problematic outcomes in alveolar echinococcosis control

Control measures for *E. multilocularis* have concentrated mainly on targetting the adult parasite by deworming or culling of fox hosts. Different characteristics of *E. multilocularis* make the control of this parasite very challenging. The parasite has very endurable eggs that can survive in the environment for long periods, and it can survive also in a wide range of small mammal intermediate hosts without being affected by deworming and/or culling measures (Veit et al., 1995; Federer et al., 2015). Regardless of these difficulties, different studies have proven the feasibility to control this parasite and thus to significantly lower the infection pressure for human AE (Hegglin and Deplazes, 2013). However, the outcomes between the different studies differ strongly. Whereas some baiting experiments had only minor or no effect on the parasite abundance in some study plots (Antolova et al., 2006; Comte et al., 2013), other baiting trails were successful to lower the prevalence in foxes and the environmental contamination with *E. multilocularis* eggs to a very low level (Schelling et al., 1997; Hegglin et al., 2003; Koenig et al., 2008). In the case of Rebun Island it was even possible to remove the parasite by eradicating the local fox population (Ito et al., 2003a). However, this example refers to an atypical situation. Foxes are a non native species and their removal was not only accepted but also feasible due to the limited size of this isolated island where no foxes could immigrate from surrounding areas.

The different PZQ baiting studies differed considerably in respect to the size of the baiting area, the bait density and baiting frequency. Interestingly it was possible to reduce the abundance of the parasite strongly in most studies even on very small scale baiting areas ($\leq 6\ m^2$), with low baiting density ($\leq$20 baits/km2) and with baiting periods lower than the prepatency period of *E. multilocularis* (Table 5). However, the different studies give evidence that better results can be achieved when bait density is high and urbanized areas with high fox densities are included in the baiting areas (Koenig et al., 2008; Hegglin et al., 2003), when baits are distributed over long periods and in monthly intervals (Koenig et al., 2008; Hegglin and Deplazes, 2008). Failures to control the parasite were attributed to the presence of species that compete for the baits (e.g., wild boars, Antolova et al., 2006) and by the immigration of infected foxes into baiting areas of limited size (Comte et al., 2013). It is noteworthy that even in a small scaled baiting urban area of only 6 km^2 a persistent low contamination has been detected, even 3 years after the discontinuation of an intensive baiting programme. It was supposed that this small scale effect could be a result of the special urban situation with a high fox density and a low hunting pressure on the fox population, which could explain a low spatial dynamic within the fox population (Hegglin and Deplazes, 2008).

Regardless of these successful studies, it is questionable to what extent such control programmes can be implemented on a long term and over larger areas. The resilience of the parasite makes it very unlikely that a sustained elimination of the parasite is feasible. It has been shown that from a purely economic point of view such measures can only be cost-effective if they are pursued for several decades and concentrate on restricted areas, which are most relevant for the transmission of *E.multilocularis*, such as highly endemic areas in densely populated zones (Hegglin and Deplazes, 2013).

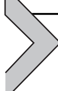
7.4 Integrated control

As outlined the transmission dynamics of *E. multilocularis* is affected by many different factors that can vary from region to region (e.g., rodent communities, agricultural practices, fox and dog densities, sanitary conditions, bait competitors), and it is unlikely to control the parasite by relying only on deworming programmes. Also the control of wildlife host populations is very challenging, linked with animal-welfare problems and at some point

also questionable from an ecological point of view (Hegglin et al., 2015). Therefore it is suggested that all control programmes should be based on a detailed knowledge of the regional peculiarities and integrate different measures in a holistic approach (Hegglin and Deplazes, 2013). An important component is to improve the awareness and give specific advice how the personal risks can be lowered (Ito et al., 2003a; Hegglin et al., 2008). Thereby it has to be considered that the risk varies strongly within the population with much higher risks for certain groups such as people working in agriculture or owning dogs (Kern et al., 2004; Piarroux et al., 2013; Wang et al., 2006a). Such groups have to be clearly defined and specifically addressed in any information campaign.

An important baseline for the implementation of an integrated control strategy is a detailed knowledge about the occurrence and ecology of the intermediate and final hosts (Deplazes et al., 2004; Liccioli et al., 2015; Raoul et al., 2015). Knowing the importance of different intermediate and final host species for parasite transmission in a given region and an understanding how their population dynamics are affected by wildlife management measures and agricultural practices are fundamental to develop regionally adapted control and prevention measures (Giraudoux et al., 1997, 2002).

In areas where AE and CE are coendemic various control measures that target owned and stray dog populations can act to lower the risk of both diseases.



8. CONCLUSIONS AND FUTURE PROSPECTS FOR CONTROL OF ECHINOCOCCOSIS

The global burden of human CE remains significant in the early part of the 21st century. In addition, although human AE is a globally rare disease there remain significant hotspots of transmission in Eurasia. The WHO has added echinococcosis to a list of 17 neglected tropical diseases, and it is prominent in the list of 12 NZDs (WHO, 2010a,b). Control and prevention of NZDs is difficult especially when treatment of humans has no ability to interrupt transmission as is the case for echinococcosis; furthermore dog, fox and livestock hosts are generally asymptomatic, and the effects of CE on livestock health is chronic and of perceived low priority (Craig et al., 2007a). The main reservoir animal hosts that sustain AE transmission are wildlife and thus hard to target practically and ethically (Hegglin et al., 2015).

Despite this, a number of intervention programmes since the 1960s have shown that the transmission of *E. granulosus* can be controlled effectively and human CE eliminated or significantly reduced as a public health problem in both island (Gemmell et al., 2001) and continental settings (Larrieu and Zanini, 2012). Successful CE control required government support with a veterinary sector as the key to delivery, long-term commitment and ability to deliver dog dosing with PZQ at a frequency of at least four times per year. Hydatid control programmes directed to resource-poor pastoral communities and semi-nomadic regions have fared less well (Lembo et al., 2013). In future, especially with global warming, such marginal regions will likely be the main zones of endemicity (Atkinson et al., 2013).

Control of transmission of *E. multilocularis* has been shown to be costly and difficult to sustain but readily achievable through targeting red fox populations using PZQ baits as indicated in several European and Japanese endemic areas including both rural and urban settings (Hegglin and Deplazes, 2013). Therefore the multifaceted human–wildlife interactions that affect the population dynamics of intermediate and final host communities should always be included in the assessment of any intervention and prevention strategy (Hegglin et al., 2015). Furthermore a landscape ecology approach to identify key host species and to understand behaviour and monitor small mammal population changes have provided robust spatial models that can help in the prediction of where human communities are at higher risk of AE disease whether in Europe or Eurasia (Giraudoux et al., 2013b). In addition the growing evidence for the role of dogs in zoonotic risk of AE disease especially in China and Central Asia can be used favourably by dosing dogs in AE endemic as well as CE/AE coendemic areas.

The development of a livestock vaccine (EG95) to prevent CE infection (Lightowlers, 2006) and of copro-tests for screening and specific identification of infected definitive hosts (Allan and Craig, 2006) have been the most important developments to aid control programmes since the discovery of PZQ in the 1970s. Integrated use of the EG95 vaccine and PZQ dosing still requires full assessment (Torgerson and Heath, 2003; Lightowlers, 2012). Future effective vaccines for echinococcosis in definitive hosts would be a major game changer for control of CE and AE (Zhang et al., 2014).

Human CE and AE cases should have access to optimal treatment, especially in resource-poor settings, for control initiatives to be viable and accepted in endemic communities. The potential to combine treatment and control measures for echinococcosis in an integrated way with other zoonotic diseases and other human and animal health issues (i.e., 'One Health'

approaches) should optimize delivery and cost benefits (Zinsstag et al., 2006; Rabinowitz et al., 2013). Such intervention approaches that include echinococcosis still remain to be undertaken.

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CHAPTER THREE

Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals

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Abstract

Among the species composing the genus *Echinococcus*, four species are of human clinical interest. The most prevalent species are *Echinococcus granulosus* and *Echinococcus multilocularis*, followed by *Echinococcus vogeli* and *Echinococcus oligarthrus*. The first two species cause cystic echinococcosis (CE) and alveolar echinococcosis (AE) respectively. Both diseases have a complex clinical management, in which laboratory diagnosis could be an adjunctive to the imaging techniques. To date, several approaches have been described for the laboratory diagnosis and followup of CE and AE, including antibody, antigen and cytokine detection. All of these approaches are far from being optimal as adjunctive diagnosis particularly for CE, since they do not reach enough sensitivity and/or specificity. A combination of several methods (e.g., antibody and antigen detection) or of several (recombinant) antigens could improve the performance of the adjunctive laboratory methods, although the complexity of echinococcosis and heterogeneity of clinical cases make necessary a deep understanding of the host—parasite relationships and the parasite phenotype at different developmental stages to reach the best diagnostic tool and to make it accepted in clinical practice. Standardization approaches and a deep understanding of the performance of each of the available antigens in the diagnosis of echinococcosis for the different clinical pictures are also needed. The detection of the parasite in definitive hosts is also reviewed in this chapter. Finally, the different methods for the detection of parasite DNA in different analytes and matrices are also reviewed.

1. INTRODUCTION

Cystic echinococcosis (CE) and alveolar echinococcosis (AE) are important helminthic zoonotic diseases caused by the infection with *Echinococcus granulosus sensu lato* (s.l.) and *Echinococcus multilocularis*, respectively. In humans and animals (intermediate hosts), CE and AE are chronic diseases caused by the growth of cysts in different organs. Prognosis of AE and especially of CE depends on multiple factors (e.g., cyst number and stage, cyst location, etc.), making the management of these diseases complex. Due to this complexity, diagnosis and followup of patients is still a matter of concern, due to several pitfalls related with the available adjunctive methods used to support the findings of imaging techniques. For CE, these adjunctive methods are mainly based on the detection of serum antibodies against crude parasite extracts (hydatid fluid; HF), but the HF contains cross-reactive antigens giving rise to false-positive results with an ample number of other parasitic and nonparasitic diseases. Some components of the HF are also responsible of nonspecific reactions with some samples from healthy donors. Additionally, a variable percentage of

patients remain serologically negative against the HF in spite of suffering CE, most probably due to clinical variables related with cyst stage, number and size, among others. The advent of recombinant techniques resulted in the definition of several recombinant antigens derived from *E. granulosus* (and *E. multilocularis*) potentially better for diagnosis than the HF. Some synthetic peptides have been also assayed for the detection of specific antibodies in patients. Some of these new reactives have been characterized regarding their usefulness in the different clinical pictures that a clinician can encounter when diagnosing a CE patient, including their performance depending on the cyst number, location and stage. For AE, the detection of specific antibodies is of higher value than for CE patients, by using specific molecules of the laminated and germinal layer, and also several recombinant antigens derived from HF components.

The same problems are faced when detection of antibodies in infected intermediate hosts is attempted. In this case, animals tend to be low responders to the hydatid cysts, and the serodiagnosis is also hampered by similar coexistent parasites giving rise to false positive reactions.

Due to the pitfalls encountered in the detection of antibodies, several alternatives for the laboratory diagnosis of patients and animals have been developed, including the detection of circulating antigens in various biological samples, the detection of cytokines in peripheral blood either with or without cell stimulation, and with less success, the assays based on the lymphoproliferation of patients' cells. Detection of circulating antigens should be accompanied in some instances by the pretreatment of the biological sample to break the antigen–antibody complexes in the sample and make antigens available for their detection. Antibodies used for the detection of antigens have been also applied for the detection of parasite molecules in cyst biopsies, aiding in the diagnostic process of patients. This parasite-extracted material has been also used for the specific amplification of parasite DNA, in order to confirm the diagnosis and to define the parasite genotype.

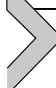
Many CE and AE cases are asymptomatic for years, and its diagnosis is still challenging due to the absence of pathognomonic signs. For this reason these diseases are frequently underdiagnosed and detected only when complications arise or by chance. When CE is detected by ultrasonography in population screening studies, tools for the detection of specific antibodies have been shown to be of low or no use, showing a high percentage of both false negative (due to a poor correlation between positive imaging and serology) and false positive results. The combination of some of the

described recombinant antigens could result in a more accurate serodiagnosis of those patients detected in population screenings.

Additionally, the clinical management of CE (i.e., surgery, percutaneous treatment and/or chemotherapy) has many associated risks for relapses, pointing out the importance of the followup of patients. Similar problems are encountered when the detection of antibodies is used for this followup, since the humoural response against the HF persist over long time periods after cure. Several recombinant antigens and cytokines have shown potential to overcome this problem. Potentially, detection of circulating antigens could be also of help for followup, but a percentage of patients lack detectable antigens regardless their clinical status.

For their transmission, the *Echinococcus* worms develop in definitive hosts, in which detection of the parasite is also of importance. The diagnosis of *E. granulosus* and *E. multilocularis* can be similarly approached by the detection of antibodies and more frequently antigens and parasite DNA in faeces. These approaches have also their limitations, covered in Sections 3 and 4 of this chapter.

In this chapter, we present the latest advances in the field of the laboratory diagnosis of *Echinococcus* spp., summarizing the best available procedures to reach an accurate detection of these parasites in different hosts and samples.



2. ANTIGENS OF *ECHINOCOCCUS* spp.

2.1 Antigens of *Echinococcus granulosus*

2.1.1 Native antigens

The hydatid cyst (metacestode) of *E. granulosus* is a complex organism constituted by several components from which different antigens are derived. Cysts are fluid-filled vesicles limited by two layers. The outermost laminated layer (LL) is of variable thickness, and it is an acellular coat secreted by the inner germinal layer. The LL protects the inner structures of the cyst by allowing the passage of molecules up to 150 kDa, but precluding the passage of cells, thereby protecting the cyst against host cellular responses (Díaz et al., 2011). The germinal layer is the cellular and proliferative sheet of the cyst, and produces brood-capsules where protoscoleces are contained. Cell composition of the germinal layer is complex, including undifferentiated cells, muscle cells, storage cells and others.

The LL is a structure rich in carbohydrates, constituting a specialized extracellular matrix that is only found in *Echinococcus*. The LL begins to be secreted by the oncosphere when reaching a target organ in the intermediate host, being in these first steps of development similar to the mature LL in fully developed cysts already at 14–20 days post infection (after egg ingestion; Holcman and Heath, 1997). The structure consists of fibrils and dense granules, the later only present in the cysts of *E. granulosus*. The fibrils are mainly composed of highly glycosylated galactose-type glycoproteins, N-acetylgalactosamine and N-acetylglucosamine, called mucins, with particular glycosylated structures (Díaz et al., 2015). Dense granules are composed by calcium inositol hexakysphosphate, which is believed to act as a store of calcium and phosphate. Besides these components, the matrix of the LL further contains other proteins adsorbed onto its structure that are mainly derived from the host.

The LL plays an important role in the triggering of immune response of the host. As mentioned, as early as 14–20 days after infection, cells of the innate immunity contact the LL compounds, resulting in the generation of noninflammatory and/or suppressor phenotypes (Díaz et al., 2009). This leads the immune system of the host to a tolerogenic phenotype that is maintained throughout the establishment and further development of the cyst. This result in the so-called modified Th2 response maintained and regulated by $CD4^{+}FoxP3^{+}$ and $CD8^{+}FoxP3^{+}$ regulatory T cells (Treg). The modified Th2 response is also of importance for the regulation of humoural responses triggered by $CD8^{+}FoxP3^{+}$ Treg cells while the cyst maintains intact its LL, which is mainly driven by the IgG4 subisotype (Pan et al., 2013). It is known that liver-specific cells that are involved in innate immunity have specific receptors on their surface for the carbohydrates found in the LL, and thus Treg-induced responses are also induced and amplified to other organs (Díaz et al., 2015).

Therefore, and although during the onset of infection local inflammatory responses may occur against the precyst, the resolution of the inflammation occurs during the establishment of the cyst, resulting in chronic stages in the formation of an adventitial layer of host origin mainly composed by collagen. The resolution of inflammation, a crucial point for parasite survival, is concomitant with the production of the LL due to the recognition by the innate immunity of the antigens of the LL. Nevertheless, carbohydrate antigens from the *E. granulosus* LL have not been characterized regarding its usefulness as diagnostic antigens.

Glycosylation is also found in many other 'internal' antigens of the cyst, constituting a group of highly immunogenic moieties but with low specificity regarding responses directed against the glycosylated component of those antigens, since these components are widely represented in many infectious and noninfectious cells. Thus, *E. granulosus* carbohydrates are responsible for immunological cross-reactions with sera from patients infected with other helminths (Sterla et al., 1999). Some examples of these other glycosylated epitopes are the P1 epitope of the blood group, present in protoscoleces and the LL (Makni et al., 1992), and the Tn carcinoma-associated antigen, capable of evoking both humoural and cellular immunity, present in adult worms and cysts (Alvarez-Errico et al., 2001) and widespread expressed in helminth parasites (Casaravilla et al., 2003). The Ag5 also presents oligosaccharides that are the major immunodominant determinants of this antigen (Lorenzo et al., 2005).

As mentioned, the cyst is filled by a fluid called HF. The HF is a complex mixture of glyco- and lipoproteins, carbohydrates and salts derived from the parasite metabolism. Some of its components are internalized from the host, mainly serum albumin and immunoglobulins. The best characterized, more abundant and more immunogenic antigens of the HF are the antigen B and the antigen 5.

The HF is to date the main antigenic source in serological tests for the detection of antibodies in patients affected by CE. However, it cannot be obtained in the laboratory and has to be collected from cysts in naturally infected animals or patients. In this sense, its composition largely depends on its source, mainly due to the variability among parasite phenotypes infecting different hosts and among different cystic developmental stages (Aziz et al., 2011; Ahn et al., 2015a). Other variables affecting the HF composition are, e.g., cyst integrity and cyst fertility. Notably, with respect to the HF variability among cyst stages, a recent work based in the comparative proteomic study of the antigenic composition of the HF in two defined cyst stages [CE1 and CE2, following the WHO Informal Working Group on Echinococcosis (WHO-IWGE) cyst classification], showed that some defined antigens are more abundant in one stage than another (Ahn et al., 2015a). For example, the whole content of AgB in CE2 cysts is higher than in CE1 cysts, although the ratio AgB1/AgB2 is different between the two stages, being AgB1 more abundant in CE1 than in CE2 cysts. Similarly, the Ag5 is more abundantly expressed in CE2 cysts compared to CE1 cysts (Ahn et al., 2015a). Therefore, the HF is difficult to obtain and it is heterogeneous among different sources.

Native antigens derived from HF have also been described, and mainly correspond to semipurified fractions enriched with Ag5 and/or AgB. The AgB, described in the 1970s (Oriol and Oriol, 1975), is a highly immunogenic protein polymer of 120–160 kDa that dissociates under reducing conditions into subunits of 8, 16 and 20–24 kDa, suggesting that it is composed of multimers of 8 kDa (Lightowlers et al., 1989). The biological role of this antigen is not fully known, but could be involved in modulating the host immune responses. In this sense, it has been characterized as a protease inhibitor, inhibitor of neutrophil chemotaxis, and promoter of Th2 nonprotective responses, inducing apoptosis of immune cells in patients with active cysts (Shepherd et al., 1991; Mamuti et al., 2006a). Relatively recent research suggests that AgB could also be responsible for detoxification mechanisms by sequestering xenobiotic compounds (Cui et al., 2013).

Molecular studies have shown that the AgB is encoded by a multigenic family, with at least five genetic groups called AgB1 to AgB5, although recent genetic studies showed that isoforms 3 and 5 probably represent the same isoform (Fig. 1) (Mamuti et al., 2006a). The five subunits differ from one another between 44% and 81% in its amino acid sequence, and the change of expression of one isoform to another has been proposed as a novel mechanism for immune evasion. The five subunits of the AgB are

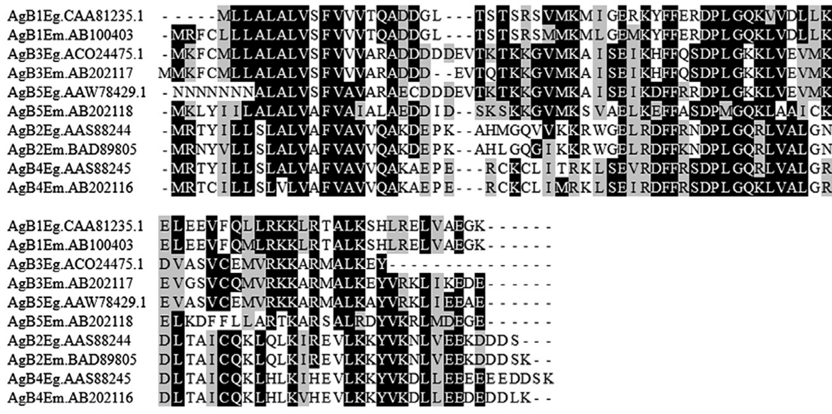


Figure 1 Comparison of the amino acid sequences of the five AgB subunits in *Echinococcus granulosus* and *Echinococcus multilocularis*. The alignment was done with Boxshade (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::boxshade). Threshold (fraction of residues that must be identical or similar for shading to occur) = 0.50. *Eg*, *E. granulosus*; *Em*, *E. multilocularis*. GenBank accession numbers are shown for each sequence.

also expressed by the related species *E. multilocularis*s. In this species, isoforms 1 to 4 are very similar at the amino acid level (over 90% of homology), whereas the isoform AgB5 is the most different from the other isoforms (Tsai et al., 2013). For both species, the different AgB isoforms are differentially expressed in different parasitic stages (cysts, adult worms and protoscoleces; Zhang et al., 2010). Regarding the degree of conservation of the different isoforms between these two species, this is high for all the subunits and higher than the homologies among different isoform in a given species (Fig. 1). Additionally, antigens similar to AgB are also found in parasites from the genus *Taenia*, including *Taenia solium* and *Taenia saginata* (Olivo et al., 1988).

The Ag5 is also a highly abundant and immunogenic antigen from the HF. Ag5 is a thermolabile protein of around 400 kDa in weight, composed of subunits of 57 and 67 kDa that dissociate under reducing conditions in subunits of 38 and 22–24 kDa (Lightowlers et al., 1989). Studies on the N-terminal sequence of the 38 kDa subunit of the Ag5 have shown that different isoforms of the same subunit are present, and therefore Ag5 may be encoded by a multigene family similar to AgB, although this hypothesis should be confirmed (Zhang and McManus, 1996). The biological function of this antigen is largely unknown, although its high concentration in HF suggests that it has important functions in the cyst development. In this sense, several studies have indicated that the 38 kDa subunit is related with the serine family of trypsin proteases, but the catalytic serine at position 192 is absent, and no peptidase activity has been found for this molecule (Lorenzo et al., 2003). The 22 kDa subunit has heparan-sulfate proteoglycans and calcium binding sites, suggesting that this subunit interacts with the cell surface and the extracellular matrix (rev. in Siracusano et al., 2012). Similar to AgB, the Ag5 is also expressed by *E. multilocularis* and *Taenia* spp., showing high homology with the Ag5 sequence found in *E. granulosus*.

Semipurified fractions enriched in AgB and/or Ag5 can be obtained from HF by different means, including molecular weight exclusion chromatography, immunoaffinity purification and others. Nevertheless, obtainment of semipurified antigens from this source has not been standardized. This, together with the intrinsic composition variation of HF from different sources, has precluded the description of a widely accepted approach for the obtainment of a homogeneous product. In this sense, a recent publication by Pagnozzi et al. (2014) described a method based in exclusion chromatography for the obtainment of an Ag5-enriched fraction from

different HF sources that resulted in similar products regardless the heterogeneity of the starting material. Nevertheless, it should be mentioned that the yield of this procedure is not detailed.

In summary, the HF and their native purified fractions are collected from infected animals (cannot be obtained in the laboratory), are therefore heterogeneous, and contain carbohydrate epitopes, resulting in false positive and negative outputs when used as antigen for the detection of antibodies. Similar drawbacks are encountered when somatic or excretory-secretory extracts from other sources (adult worms and protoscoleces) have been used for the detection of antibodies in patients or in intermediate hosts (Ersfeld et al., 1997; Rafiei and Craig, 2002; Carmena et al., 2004). Some purified antigens have also been obtained, among them the P29 protoscoleces antigen, described by Gonzalez et al. (2000). Extracts of oncospheres have been also obtained and used to detect antibodies in people from endemic areas (Craig, 1988). Purified proteins from the same source were also used to demonstrate specific antibodies in experimentally infected sheep (Heath and Lawrence, 1996). Nevertheless, due to the difficulty in obtaining these native antigens, no further use of oncospheral extracts have been done.

In this sense, the use of recombinant or peptide antigens could be a good alternative to native antigens, giving the possibility of reducing nonspecific reactions and allowing their standardized obtainment and diagnostic evaluation in different laboratories.

Antigens for the detection of specific antibodies in definitive hosts (dogs) have also been described. These are mainly somatic and secretory extracts from protoscoleces and adult worms. While serology in definitive hosts has proven difficult for both *E. granulosus* and *E. multilocularis*, coproantigen tests utilizing somatic extracts or excretory/secretory antigens obtained from protoscolices or adults parasites have been successfully used (for reviews, see Craig et al., 2015; Conraths and Deplazes, 2015; and Section 4 of this chapter).

2.1.2 Recombinant antigens and synthetic peptides

The recombinant proteins are produced in its vast majority in prokaryotic systems by using different strains of *Escherichia coli* as host for recombinant plasmids containing the sequence that codifies the protein of interest. Different *E. coli* strains can produce diverse modifications, resulting in products with different characteristics. Additionally, different vectors can contain different protein tags, also giving rise to different recombinant products. The

intrinsic variability of these systems can be complemented by other factors influencing the final composition of the recombinant protein, including potential modifications (e.g., deletion of signal peptides) and, in our settings, the parasite genotype from which the sequence has been obtained. In this sense, sequence variation of the same antigen among different parasite genotypes has been shown (e.g., Haag et al., 2004, 2006; Kamenetzky et al., 2005; Muzulin et al., 2008; Boubaker et al., 2014), although the extent of this variability could be low enough to result in cross-reactive antibody responses among the different parasite genotypes for some antigens (Fig. 2). More important for the potential triggering of different antibody responses is the lack of expression of defined subunits of some antigens (e.g., AgB) in some parasite genotypes. It has been shown that AgB2 is expressed by the G1/G2 genotypes, but not by the G5 and G6/7 genotypes

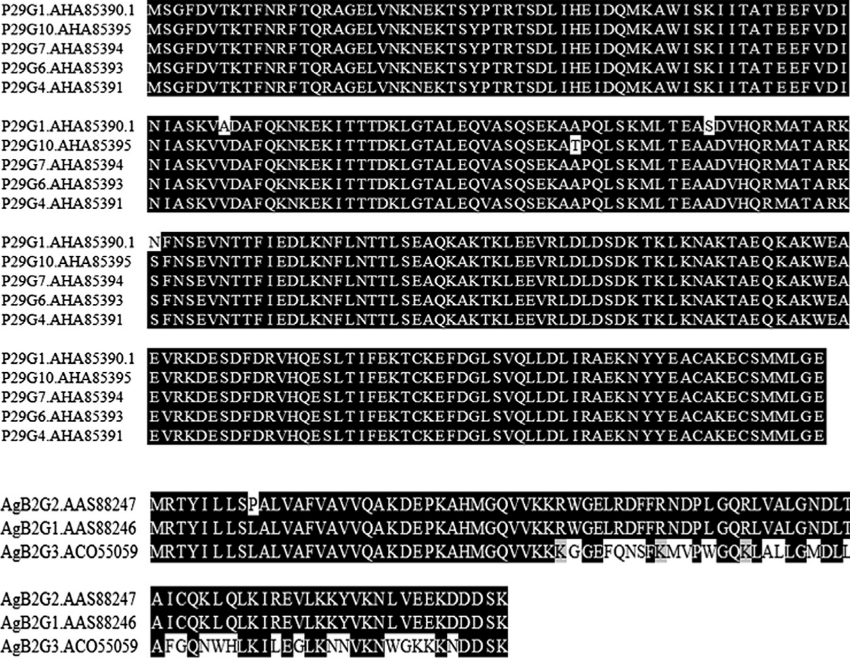


Figure 2 Variability of the antigen P29 and the antigen B2 among different *Echinococcus granulosus* genotypes. The alignment was done with Boxshade (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::boxshade). Threshold (fraction of residues that must be identical or similar for shading to occur) = 0.50. GenBank accession numbers and genotype number are shown for each sequence.

(occasionally infecting humans; rev. in Alvarez Rojas et al., 2014), these showing only AgB4 as potentially functional genes (Kamenetzky et al., 2005).

Thus, and although recombinant proteins per definition are more homogeneous (clonal) than native products, the above-mentioned factors influencing their final composition should be taken into account if a homogeneous product has to be obtained and its use has to be standardized. When its obtainment has been clearly defined, uniform recombinant antigens can be easily produced at high quantities in few hours in the laboratory.

All the isoforms of AgBs from *E. granulosus* have been produced as recombinant proteins by different laboratories, except for AgB5 (Haag et al., 2004). The first description of a sequence corresponding to AgB was done by Shepherd et al. (1991). The described sequence corresponds to a fragment of the AgB1. This was used by McVie et al. (1997) and Ibrahem et al. (2002) to generate specific primers and the corresponding cDNA was cloned and produced as truncated AgB1 recombinant protein tagged with maltose. Two sequences corresponding to the full coding frames of AgB1 (described by Frosch et al., 1994) and AgB2 (described by Fernandez et al., 1996) were cloned and produced as recombinant proteins with a glutathione transferase (GST) tag, and final recombinant products were obtained after thrombin cleavage, thus free of the GST (Rott el al., 2000). These were also produced and used by Virginio et al. (2003), Lorenzo et al. (2005) and Chandrasekhar and Parija (2009). The AgB1 was produced and used as a GST-fusion protein by Ortona et al. (2000). The AgB1 has been also produced and tested as a recombinant protein after its production in the pQE30 expression vector, resulting in a 6xhistidine-tagged protein (Kalantari et al., 2010). AgB1 and AgB2 (G1 genotype) have also been produced as truncated, untagged recombinant proteins lacking the 5'end-terminal sequence coding for the signal peptide (Hernandez-Gonzalez et al., 2008). The same sequence of AgB2 was used for the recombinant production of a head-to-tail tandem repeat protein, called 2B2t (Hernandez-Gonzalez et al., 2012).

The coding sequence for AgB3 was first described by Chemale et al. (2001) and further used to produce the corresponding recombinant protein (Monteiro et al., 2007) by using the same approach than Rott et al. (2000) — a GST-tagged protein further cleaved with thrombin-. The AgB4 was described by Arend et al. (2004). Truncated versions of AgB1, AgB2, AgB3 and AgB4 were obtained as recombinant proteins fused with thioredoxin and assayed by Jiang et al. (2012a,b). The AgB5 was described by

Haag et al. (2004) but has never been produced as recombinant protein. Thus, methods for the obtainment of recombinant antigens derived from *E. granulosus* among different laboratories have not reached a consensus, resulting in different recombinant proteins that preclude their comparison regarding their diagnostic performance among different laboratories.

A second derivative of native antigens is represented by synthetic peptides. The immunogenicity and thus potential for diagnosis of these molecules can be studied by clone reactivity with specific antibodies and by phage display or predicted by bioinformatics. Synthetic peptides represent a better alternative than recombinant antigens with respect to their definition and standardization, due to their chemical obtainment following a defined sequence of amino acids. Nevertheless, these are linear epitopes and the potential conformational epitopes with importance in the triggering of immune responses in the host cannot be obtained by these means, so this option is more limited than the obtainment of recombinant antigens in this aspect.

Several synthetic peptides derived from the AgBs with diagnostic potential have been described in the literature (Fig. 3). Leggatt and McManus (1994) described a 27 amino acids long synthetic peptide (peptide 65), representing an epitope in the N-terminal region of the AgB1 (amino acids 28–54). Residue modification of p65 and respective reactivity was further studied by Barbieri et al. (1998), Gonzalez-Sapienza et al. (2000) and González-Sapienza and Cachau (2003). Their findings showed that the antigenic determinants built by the stretch EVKYFFER are major B cell epitopes, proving to be a major immunodominant region of the AgB1 (González-Sapienza and Cachau, 2003).

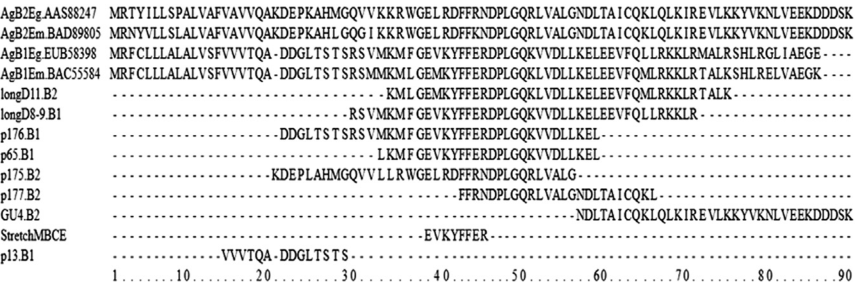


Figure 3 Peptides of the antigen B1 and antigen B2 of *Echinococcus granulosus* and *Echinococcus multilocularis* obtained by different authors.

The peptide p176, also derived from AgB1 (38-mer, from amino acids 17–54), was first described by Gonzalez-Sapienza et al. (2000) and further tested by González-Sapienza and Cachau (2003) and Santivañez et al. (2012). Gonzalez-Sapienza and Cachau (2003) described an additional peptide of AgB1 called p13 and containing the amino acids 15 to 28 of AgB1.

From the AgB2, the peptide GU4 was described by Barbieri et al. (1998) and corresponds to a 34-mer peptide covering the C-terminal end of the AgB2 (amino acids 57–90). This was further assayed by Gonzalez-Sapienza et al. (2000). These authors described two additional peptides from AgB2: p175 (36-mer, amino acids 21–56) and p177 (25-mer, amino acids 42–66).

In an attempt to find additional peptides with immunodominant characteristics from different *E. granulosus* antigens, List et al. (2010) performed an explorative selection of diagnostic peptides by bioinformatics and peptide microarray validation. Sequences of the parasite present in US National Center for Biotechnology Information (NCBI) were elected based on their alpha-helical coiled-coil (CC) and antigenicity (IUR) predictions. Detected regions of high predicted CC stability or antigenicity and increased solubility were favoured for the definition of 30-mer peptides. Of the 45 peptides selected, 8 showed to be the most reactive against sera from alveolar and cystic echinococcosis patients. From these, 3 derived from the following *E. granulosus* antigens: AgB1 (peptide longD8-9, amino acids 29–70), AgB2 (peptide longD11, amino acids 46–90) and the protoscoleces antigen EG19 (DeLunardo et al., 2010) (peptide longD3, amino acids 1 to 42). These antigens were pointed out experimentally to have diagnostic potential by other authors in earlier publications, thus the bioinformatic analysis performed by List et al. (2010) confirmed the usefulness of this approach for the definition of diagnostic peptides as an alternative to peptide selection by antibodies, reducing complex lab work.

The Ag5 has also been produced as a recombinant protein. Lorenzo et al. (2005) produced two versions of this antigen: a long one, covering amino acids 22 to 484 without the leader peptide, and a short one, corresponding to the 38 kDa subunit of the Ag5 (amino acids 189–484). The long version was expressed as a histidine-tagged protein, while the 38 kDa subunit was expressed as a fusion protein with maltose binding protein (Lorenzo et al., 2005). Later, Li et al. (2012) produced the 484 amino acids long full-length Ag5 recombinant protein as a histidine-tagged protein. The several available versions of the recombinant Ag5 are different and thus not comparable regarding their immunoreactivity.

Only one synthetic peptide, called p89-122, was described as a major epitope of Ag5 (Chamekh et al., 1992), but later shown to be a fragment of a 29 kDa protoscolex component of *E. granulosus* (Gonzalez et al., 2000).

Additional recombinant antigens different from AgB and Ag5 have also been described. These include antigens from protoscoleces, oncospheres and adult worms. Ag4 or malate dehydrogenase (MDH), the cystosolic enzyme from protoscoleces, was first described by Rodrigues et al. (1993) and obtained as a GST-tagged recombinant protein by Ferreira and Zaha (1994). This antigen clone was elected due to its reactivity with CE patients' sera. The same recombinant antigen but without the GST tag, was further produced by Virginio et al. (2003) and Lorenzo et al. (2005). This antigen is very similar to that expressed by the related species *E. multilocularis* (>90% identity). A molecule with some similarity to the *E. granulosus* MDH is also found (<50% identity) in parasites of the *Taenia* genus.

Virginio et al. (2003) produced three additional recombinant antigens representing different proteins of protoscoleces: calcium binding protein (CaBP; Rodrigues et al., 1997), actin filament fragmenting protein (AFFP; Cortez-Herrera et al., 2001) and a truncated version of AFFP (amino acids 261–370). All were produced as GST-tagged proteins and further cleaved with thrombin to obtain the antigens without the GST tag (Virginio et al., 2003). The CaBP is present in cestodes with calcareous corpuscles, and thus present in the genus *Taenia* and in *E. multilocularis* (>90% homologous with the CaBP from *E. granulosus*). AFFP is also present in both *E. multilocularis* and *Taenia* spp, showing a high homology in its sequence among these different parasites.

A pool of serum samples from mice infected with oncospheres of *E. granulosus* was used to screen a cDNA library constructed with RNA extracted from protoscolex from sheep hydatid cysts. A clone was elected and the corresponding GST-tagged recombinant protein (EpC1-GST) was obtained by Li et al. (2003). Later, the same authors (Li et al., 2004) obtained two additional protoscoleces recombinant antigens by using the same approach: thioredoxin peroxidase (first described by Salinas et al., 1998) and EgG5. These were also obtained as GST-tagged recombinant proteins. Both are also expressed by *E. multilocularis* and *Taenia* spp., showing high homology with the corresponding proteins of *E. granulosus* (<90%). A second thioredoxin peroxidase His-tagged recombinant antigen was obtained by Margutti et al. (2008), showing 99% identity with that obtained by Li et al. (2004) and probably representing the same molecule.

Later, Zhang et al. (2007) identified a specific antibody-binding region of EpC1 One peptide designated peptide 5-P5, fused with GST, was positively recognized by sera from mice experimentally infected with oncospheres of *E. granulosus* and sera from surgically confirmed CE patients. This peptide represents amino acids 131 to 156 of the full-length antigen.

Several other recombinant proteins from *E. granulosus* somatic compounds have proved their potential as diagnostic antigens and have been usually identified and cloned after screening of expression libraries with sera from CE patients. The antigen EgTeg has been produced as a His-tagged recombinant protein by Ortona et al. (2005), representing an N-terminal truncated sequence of a tegumental protein of protoscoleces. Two additional proteins from protosocoleces related with allergic responses (IgE) in CE patients were also identified and produced as His-tagged recombinant proteins by this group: an N-terminal truncated heat shock protein (HSP70; Ortona et al., 2003) and the full-length elongation factor-1 beta/delta (EgEF-1 β/δ) together with three truncated versions of the same protein (Margutti et al., 1999; Ortona et al., 2001). The EgG1Y162 protein from protosocoleces has been also cloned and expressed as a His-tagged recombinant antigen (Zhang et al., 2014), showing 70% identity with the oncosphere antigen B (Zheng et al., 2013) and 40% identity with the EG95 antigen. Paramyosin has been cloned and produced as a His-tagged recombinant protein by Moghadam et al. (2013). The P29 His-tagged recombinant antigen has been obtained from both G1 and G6 *E. granulosus* genotypes, showing minor differences in their amino acid composition without measurable impact on P29 antigenicity (Ben-Nouir et al., 2009; Boubaker et al., 2014). Finally, the truncated antigen E14t, corresponding to the 14-3-3 zeta protein of the parasite (Siles-Lucas et al., 2003) and the C317 antigen, a protein from the germinal layer of cysts (GenBank nb. BI244032) were produced and used a recombinant antigens after thrombin cleavage of their GST tag (Hernández-González et al., 2008). All these antigens are also expressed by *E. multilocularis*, with homologies ranging from 80% to 95% with the corresponding sequences in *E. granulosus*. The EG95 recombinant antigen derived from oncospheres has also been obtained as a potential diagnostic antigen as a GST-fused protein (Lightowlers et al., 1996).

Recombinant antigens and synthetic peptides from *E. granulosus* that have been used either for detection of antibodies in dogs or for the production of polyclonal or monoclonal antibodies to detect antigens in infected dogs are covered in Section 4 of this chapter.

A summary of the available native, semipurified, recombinant and peptide antigens from *E. granulosus* with diagnostic potential is shown in Table 1.

2.2 Antigens of *Echinococcus multilocularis*

2.2.1 Native antigens

Since human AE is caused by metacestodes of *E. multilocularis*, the development of serological tests for diagnosis of AE was largely focussed on this larval stage as source for diagnostic antigen production. Metacestodes are fluid-filled vesicles that are delineated by an inner cellular tissue (germinal layer) and by an outer acellular tegument (LL) (e.g., reviewed by Gottstein and Hemphill, 2008). Both germinal and LL are of parasite origin. However, while protoscolex formation from the germinal layer is common for the natural rodent intermediate hosts, this has been rarely reported in human cases (Kern, 2006; Gottstein, 1992). For this reason, vesicular fluid (Walker et al., 2004; Müller et al., 2007) as well as the germinal and LLs of the cyst wall (Mamuti et al., 2004; Ingold et al., 1998, 2001), represent major sources of antigenic peptides and/or carbohydrates of *E. multilocularis*.

In humans infected with *E. multilocularis*, the metacestode stage forms cystic, or rather vesicular, infiltrates appearing as conglomerates of small lesions inside the liver tissue (Vuitton and Gottstein, 2010; Gottstein et al., 2015). Metacestode vesicles proliferate asexually via formation and budding of daughter vesicles. This tumour-like growth leads to an accumulation of a heterogenous parasitic mass in the liver that exhibits proliferative characteristics in the periphery and often becomes necrotic in its centre (Vuitton and Gottstein, 2010; Gottstein et al., 2015). Probably due to both its infiltrative growth behaviour and the lack of fibrous tissue encapsulation induced by the host inflammatory response to the infection (a phenomenon characteristic for *E. granulosus* hydatid cysts, see previous sections), *E. multilocularis* metacestodes are exposed to the host's immune system and thus elicit a strong cellular and humoural immune response in the infected individuals. During an *E. multilocularis* infection, immunological events preferentially occur at the host—parasite interface and include formation of periparasitic granuloma composed of macrophages, T cells and myofibroblasts (Vuitton et al., 1989; Vuitton and Gottstein, 2010; Gottstein et al., 2015). Immunological studies suggest that the maintenance of a Th1-oriented cellular immune response considerably affects the ability of the parasite to establish infection (Gottstein et al., 2015). Indeed, a previous study demonstrated a significant increase of the AE incidence in

Table 1 Summary of the available native, semipurified, recombinant and peptide antigens from *Echinococcus granulosus* with diagnostic potential in human cystic echinococcosis and range of specificity and sensitivity in enzyme linked immunosorbent assay[a]

Antigen	Characteristics	Sensitivity range	Specificity range
Native			
Hydatid fluid	Crude	64.8–100%	40–100%
Antigen B	Semipurified	60–96.9%	77–100%
Antigen 5	Semipurified	50 to 100%[b]	89 to 100%[b]
Protoscoleces	Somatic	69.4–96.9%	48–57%
Others[c]	Somatic	81.3–96.7%	65–93%
Recombinant			
Antigen B1	Several versions[d]	55–94.6%	80–91%
Antigen B2	Several versions[e]	45–93%	86–98%
Antigen B3	Several versions[f]	29%	76.5%
Antigen B4	Truncated, thioredoxin tag	75.8%	73.1%
Antigen 5	Aa. 22 to 484, His-tag	65%	89%
Antigen 5 (38 kDa)	Aa. 189 to 484, MBP-tag	21%	97%
Antigen 5	His-tag	NT	NT
MDH (Ag4; cytosolic)	Several versions[g]	45–90%	83–95%
CaBP	Full length, untagged	84%	97%
AFFP	Several versions[h]	59–69%	90–96%
EpC1	GST-tag	33–92%	95%
TrxP	Several versions[i]	39%	69%
EgG5	GST-tag	61%	70%
EgTeg	His-tag, truncated	65–73%	44–89%
HSP70	His-tag, truncated	NT	NT
EgEF-1 β/δ	Several versions[j]	NT	NT
EgG1Y162	His-tag	NT	NT
Paramyosin	His-tag	NT	NT
P29	His-tag	62.5%	NS
E14t (14-3-3z)	Truncated	35.3%	91.7%
C317	Full length	58.8%	80.9%
EG95	GST-tag	NT	NT
Synthetic peptides			
p65 (AgB1)	Aa. 28 to 54	34–48%	80–97%
p176 (AgB1)	Aa. 17 to 54	23.8–80%	83–93%
p13 (AgB1)	Aa. 15 to 28	NT	NT
GU4 (AgB2)	Aa. 57 to 90	12–18%	96–100%
p175 (AgB2)	Aa. 21 to 56	49%	94%

(*Continued*)

Table 1 Summary of the available native, semipurified, recombinant and peptide antigens from *Echinococcus granulosus* with diagnostic potential in human cystic echinococcosis and range of specificity and sensitivity in enzyme linked immunosorbent assay[a]—cont'd

Antigen	Characteristics	Sensitivity range	Specificity range
p177 (AgB2)	Aa. 42 to 66	38%	92%
p89-122 (29 kDaPps)	Aa. 89 to 122	16–85%	77–100%
GST-5-P5 (EpC1)	Aa. 131 to 156	97%	NT

[a]Rev. in Carmena et al. (2006); Sarkari and Rezaei (2015); Manzano-Roman et al. (2015).
[b]From Pagnozzi et al. (2014).
[c]Adult worms extract, cyst wall extract, protosocoleces tegument extract.
[d]Truncated, full length, tagged with maltose, GST or 6xhistidine.
[e]Full length, truncated, 2xtandem repeat.
[f]Full length, truncated tagged with thiorredoxin.
[g]Full length, tagged with GST.
[h]Full length, truncated (amino acids 261–370).
[i]GST-tag, His-tag.
[j]His-tag full length, His-tag truncated; *Aa*, amino acids; *His*, histidine; *MBP*, maltose binding protein; *NS*, not stated; *NT*, not tested; *Pps*, protoscolex.

immunocompromised people in France after 2000 (Chauchet et al., 2014). This may indicate that individuals taking immunosuppressive drugs are at high risk of infection with *E. multilocularis*. Patients with chronic AE exhibit a Th2-biased or a Th1/Th2-mixed immune response basically allowing long-term parasite survival, proliferation and maturation (Gottstein et al., 2015). In addition, the chronic stage of AE was demonstrated to coincide with both production of proinflammatory cytokines in the periparasitic granuloma and a certain restriction of parasite growth through fibrosis and necrosis (Vuitton and Gottstein, 2010; Gottstein et al., 2015). Conversely, the regressive course of AE is associated with Th1-biased protective immune reactions probably accounting for abortive AE cases carrying spontaneously healed and often calcified lesions after *E. multilocularis* infection (Vuitton and Gottstein, 2010; Gottstein et al., 2015).

While cellular immune reactions are involved in control of AE, the possible role of antibodies in stimulating anti *Echinococcus* effector functions in immune cells that recognize their Fc region remains to be elucidated. Even so, various investigations in the past revealed that the large majority of AE patients produce high amounts of antibodies, which are mostly directed against metacestodes (Vuitton and Gottstein, 2010; Gottstein et al., 2015). Accordingly, the development of serology for diagnosis of human AE was focussed from the beginning on the use of metacestode antigens either as native, or as recombinant, proteins. As described in the

following, these diagnostic antigens belong to different compartments of *E. multilocularis* metacestodes and are included in different assay systems some of which turned out to be highly valuable tools for serological detection of human AE cases (see below).

The vesicles of *E. multilocularis* metacestodes consist of an inner cellular and an outer acellular compartment (Gottstein and Hemphill, 2008). The parasite-derived, acellular surface is essentially constituted by the carbohydrate-rich LL that covers the entire surface of the vesicles. This surface structure is much less prominent than the LL covering the *E. granulosus* hydatid cysts (Gottstein and Hemphill, 1997). The LL is supposed to act as protective shield against immunological attack particularly during the early larval development of *E. multilocularis* (Vuitton and Gottstein, 2010; Gottstein et al., 2015). Interestingly, this 'immunoprotective' carbohydrate-rich layer contains an abundant antigenic structure, Em2, which was characterized as a mucin-type glycoprotein complex (Hülsmeier et al., 2002; Yamano et al., 2012). Since Em2 is a T-cell independent antigen, the respective antibody response lacks antibody maturation (Dai et al., 2001; Gottstein and Hemphill, 2008; Vuitton and Gottstein, 2010). Thus, Em2 may be exposed by the parasite as an 'inefficient' antigen that avoids successful immunological attack by the host (Dai et al., 2001).

Em2 was applied for the development of an enzyme linked immunosorbent assay (ELISA) and turned out to be essential for a reliable differential diagnosis of AE in humans (Gottstein et al., 1983, 1993; Müller et al., 2007). The dominant antigenic epitope of Em2 was identified as a trisaccharide, namely Gala1-4Galb1-3GalNAca1-R that is responsible for the excellent diagnostic sensitivity and good specificity of this carbohydrate antigen (Yamano et al., 2012). Minor limitations in specificity may be due to the terminal part from this trisaccharide, Gal1α1-4Galβ that accounts for occasional cross-reactivities with sera from patients suffering from other helminth infections. In another approach focussed on similar molecules, Galb1-6(Fucα1-3)Galβ1-6Galβ1-ceramide, a chemically synthesized carbohydrate antigen with the structure of a glycosphingolipid revealed a significant potential to serologically differentiate between AE and CE (Yamono et al., 2006).

Interestingly, Em2 also seems to contain peptidic epitopes that are shared with the *E. multilocularis* alkaline phosphatase (EmAP). The molecular, and perhaps a functional, relationship among these two antigens was substantiated by the observation that both are localized in the LL apart from additional locations of EmAP on the glycocalyx and in the central part of

the protoscoleces (Lawton et al., 1997). EmAP was revealed to be an abundant enzyme from *E. multilocularis* that is released upon damage of metacestodes as e.g., demonstrated by chemotherapeutical treatment of in vitro-cultivated vesicles from the metacestodes (Ingold et al., 1998; Stettler et al., 2001).

The inner, cellular tissue from *E. multilocularis* metacestodes contains a population of different cell types that form the germinal layer. Its outer part is attached to the inner surface of the LL (Gottstein and Hemphill, 2008). The germinal layer forms microvilli-like extensions (microtriches) that protrude into the matrix of the LL and thus increase the resorbing surface of the parasite. This structure contains undifferentiated cells but also highly differentiated cell types such as glycogen storage cells, muscle cells and connective tissue cells (Sakamoto and Sigimura, 1970; Koziol and Brehm, 2015). Release of proliferative cells from the germinal layer into the blood or lymph system leads to metastasis formation in various organs including lung, kidney and brain (Ali-Khan et al., 1983; Eckert et al., 1983; Mehlhorn et al., 1983; Vuitton and Gottstein, 2010). In small rodents acting as natural intermediate hosts of *E. multilocularis*, the internal part of the germinal layer of the metacestodes forms brood capsules where multiple protoscolices are produced by asexual division (Trouvé et al., 2003; Koziol and Brehm, 2015). In humans, however, formation of protoscolices is rarely found (see also above).

For production of native *E. multilocularis* antigen, metacestodes can be accumulated by serial intraperitoneal passages in mice (Hemphill et al., 2002). Alternatively, large amounts of host tissue-free larval material can be produced by making use of an in vitro cultivation method for the long-term maintenance and vesicular proliferation of metacestodes. Compared to the unfavourable situation in CE excluding proliferation of metacestodes in mice or in cultures, efficient production in the laboratory of *E. multilocularis* metacestodes provides an ideal basis for the extraction, purification and characterization of antigens in AE.

In the past, the cellular compartment of the *E. multilocularis* metacestodes provided two immunodiagnostically relevant native antigen fractions (Schweiger et al., 2012). These fractions were isolated from purified protoscolices and termed EmP (protoscolex Em2/cyst wall-depleted) and EmPI (protoscolex integument; Em2/cyst wall-depleted). However, the major source for crude native antigen relevant for immunodiagnosis of AE is *E. multilocularis* vesicle fluid (EmVF). As could be demonstrated for EgHF, the fluid fills the entire cyst and acts as an important nutritional matrix of

the internal environment of the metacestode (Li et al., 2013). This extracellular matrix contains polysaccharides and lipids, and is rich in proteins originating from both the parasite (e.g., antigens, alkaline phosphatase, low-density lipoprotein receptor, heat-shock proteins, different metabolic enzymes) and the host (e.g., serum albumin, hemoglobin, immunoglobulin) (Aziz, 2011; Li et al., 2013). Interestingly, here antigens constitute the largest portion of the parasite-derived proteins, antigens 5 (Ag5) and B (AgB) being by far the most abundant ones inside the metacestodes (Li et al., 2013). Due to their high content of antigens, crude preparations of EmVF became important reagents for serological diagnosis in human echinococcosis (Müller et al., 2007).

Among the different antigens identified in *E. multilocularis* metacestodes so far, and similar to what has been described for *E. granulosus*, AgB has gained a considerable diagnostic relevance in the past years (Mamuti et al., 2006a,b). Functionally, AgB was characterized as a polymeric 160 kDa lipoprotein (Oriol et al., 1971).

As mentioned for *E. granulosus*, AgB in *E. multilocularis* also consists of a group of highly immunogenic 8 kDa subunit monomers (Jiang et al., 2012a,b). AgB is encoded by a family of five genes (AgB 1–5) exhibiting a considerable genetic and antigenic variability in the corresponding 8 kDa subunits e.g., as consequence of genetic polymorphism, strain variability and differential expression of individual AgB orthologues in different developmental stages of the parasite (Mamuti et al., 2006b). This antigenic variability made it difficult to standardize AgB in terms of its suitability as an antigenic reagent in serological diagnosis of AE (and CE).

In contrast to AgB, the other abundant metacestode antigen, Ag5, has only been extensively characterized in *E. granulosus* (see above). At least, in *E. multilocularis* a protein named Em6 was identified as a homologue of Eg6 which had previously been described as an antigenic epitope of *E. granulosus* Ag5 (Siles-Lucas et al., 1998; Carmena et al., 2007). In *E. multilocularis*, native Em6 was identified as 40 kDa polypeptide and was exclusively detected in adult worms and fertile cysts of the metacestodes. However, Em6 was absent in nonfertile metacestodes. The demonstration of a protein in *E. multilocularis* displaying identities to 'antigen 6' of *E. granulosus* could potentially contribute to the future elucidation of the relationship between 'antigen 5' and 'antigen 6' in the genus *Echinococcus*.

2.2.2 Recombinant antigens and synthetic peptides

Since several years, AgB has been of wide use in the diagnosis of not only CE (see above) but also of AE. Genetic analysis revealed that the 8 kDa subunits of *E. multilocularis* AgB (named EmAgB8/1–5; Mamuti et al., 2006b) are encoded by five distinct gene loci thus exactly reflecting the genetic constellation of AgB in *E. granulosus*. While EmAgB1 to 4 share a high degree of homology (>90%) and are expressed in both metacestodes and in immature adults, EmAgB5 is less closely related (<55.7% homology) and could be detected in these two life cycle stages. As can be concluded from current literature, EmAgB1, and more specifically the recombinant version from this antigen, has been most intensely investigated regarding its potential as immunodiagnostic reagent. Recombinant EmAgB1 was produced in *E. coli* as thioredoxin/polyhistidine double-tagged protein, affinity-purified from bacterial extracts via chromatography through a resin containing bound bivalent nickel with polyhistidine binding capacity (see also below), and finally liberated from the tags by protease cleavage (Mamuti et al., 2004).

In the past, various *E. multilocularis* antigens with immunodiagnostic potential have been identified in the germinal layer. Among these, recombinant 18 kDa antigen Em18 and previously published subfragments from this antigen, namely Em II/3, Em II/3–10, EM10, and EM4 (e.g., reviewed by Carmena et al., 2007; Siles-Lucas and Gottstein, 2001), have to be mentioned in the first place. Em18 exhibits functional similarities to ERM (ezrin, radixin, moesin) and may be involved in the cellular architecture (Brehm et al., 1999). Recombinant Em18 and antigenic subfragments thereof, have gained considerable importance both in differential diagnosis of human AE and as a biomarker for serological testing the viability of an *E. multilocularis* infection in humans. Recombinant Em18 was expressed in *E. coli* as a polyhistidine-tagged protein and affinity-purified from bacterial extracts via immobilized bivalent nickel chromatography (Crowe et al., 1994; Spriesterbach et al., 2015).

Serodiagnostic characteristics similar to those of recombinant Em18 were recently attributed to a recombinantly expressed *E. multilocularis* P29 (Boubaker et al., personal communication). In the respective study, recombinant EmP29 was produced in *E. coli* as polyhistidine-tagged protein and affinity-purified as outlined in a previous section, for recombinant Em18. The P29 was first identified in *E. granulosus* (EgP29) and it was localized in protoscoleces and the germinal layer of the hydatid cyst, whereas EgP29 was absent in *E. granulosus* HF or in adult worms sections (Gonzales et al., 2000; Ben Nouir et al., 2008; Ben Nouir et al., 2009). This rather

conserved protein of as yet unknown biological function was also identified as an excretory/secretory product from in vitro-cultivated *E. granulosus* protoscoleces (Virginio et al., 2012) that turned out to be suitable as biomarker for postsurgical monitoring of CE patients (Boubaker et al., 2014). The amino-acid sequence of P29 is highly conserved within the genus of *Echinococcus* and exhibits only three amino acid substitutions between *E. granulosus sensu stricto* (genotype G1) and *E. multilocularis* (Boubaker et al., 2014). In *E. multilocularis*, EmP29 was detected in the germinal layer, in protoscolices and also in the metacestode vesicle fluid. Recombinant EmP29 demonstrated to be a diagnostically and prognostically useful tool for assessment and monitoring of AE patients (see below).

Another recombinant antigen with immunodiagnostic potential in AE, EM13, was expressed in *E. coli* as glutathione S-transferase fusion protein (Frosch et al., 1993). By using corresponding antibodies for immunohistology, EM13 was localized in the protoscolices and particularly in the microtriches on the surface of *E. multilocularis* metacestodes. EM 13 was characterized as an *E. multilocularis*-specific protein because apparently this antigen is not expressed by the larval stage of *E. granulosus* although an analogous coding sequence has been identified in the genome of the parasite (Frosch et al., 1993).

Recent developments in immunodiagnosis of AE make it evident, that the recombinant DNA technology has revolutionized both the identification and production of diagnostic antigens. Most of those recombinant antigens relevant for diagnosis of AE have been produced in *E. coli* using tag-protein fusion systems that allow the utilization of biological chemical or physical interactions to affinity purify the tagged recombinant proteins (Crowe et al., 1994; Spriesterbach et al., 2015). However, the application of recombinant antigens in serology is often hampered by residual bacterial components (such as enterotoxin) inside the purified fractions that may cause unwanted serum antibody reactions possibly leading to false positive results in the test. In order to avoid such problems, chemical synthesis of peptides may be exploited as an unlimited source for standardized production of highly pure antigen. As an initial approach, List et al. (2010) took advantage from this relatively novel concept to offer proof of principle for the identification of diagnostically relevant synthetic peptides (size 24—30 amino acids) from *Echinococcus* ssp. by bioinformatic selection and subsequent microarray-based serological screening of the preselected peptides regarding their reactivity with AE and CE patient sera. Here, peptides selected by different algorithms e.g., represented subfragments from antigens EM13,

EmII/3, EmAgB8/1 and EmAgB8/2. Initial data from this study and its limitations are presented in Section 2.1.2.

Antigens of *E. multilocularis*, both native and recombinant, and corresponding polyclonal and monoclonal antibodies for the diagnosis of definitive hosts, are covered in Section 4 of this chapter.

2.3 Antigens of *Echinococcus vogeli* and *Echinococcus oligarthrus*

Both *Echinococcus vogeli* and *Echinococcus oligarthrus* are autochthonous species in neotropical areas. *E. vogeli* is a rare finding in human patients, and *E. oligarthrus* has been found on a few occasions in infected patients (D'Alessandro et al., 1995; D'Alessandro and Rausch, 2008). The metacestode of *E. vogeli* occurs most frequently in the liver, consisting of few-to-numerous contiguous spherical vesicles. They are usually surrounded by host connective tissue and enclosed by a folded LL. Folding of the LL can result in chamber lined by germinal tissue containing brood capsules and protoscoleces. In human patients, the metacestode usually spreads from the liver into the peritoneal and pleural cavities, and numerous organs can be invaded (D'Alessandro et al., 1995). The cysts of *E. oligarthrus* are unilocular and fluid-filled, enlarging concentrically without exogenous proliferation in patients.

The LL of *E. vogeli* cysts has been investigated in in vitro-maintained metacestodes (Ingold et al., 2001), This is composed, as previously described for *E. multilocularis* metacestodes, of N-acetyl-beta-D-galactosaminyl residues and alpha- and beta-D-galactosyl residues, as well as of the core structure of O-linked carbohydrate chains, N-acetylgalactosamine-beta-1,3-galactose. Additionally, N-linked glycopeptides and alpha-D-mannosyl and/or glucosyl residues are associated with the LL of *E. vogeli*. No cross-reactivity was found when a polyclonal antiserum raised against the *E. multilocularis* LL and the monoclonal against the *E. multilocularis* antigen Em2 was tested in LL extracts of *E. vogeli*, indicating distinct compositional and antigenic differences between these two parasites (Ingold et al., 2001).

The antigens usually used for the diagnosis of patients with polycystic echinococcosis have been those, native or recombinant, described in *Echinococcus* species different from *E. vogeli* or *E. olighartrhus* (rev. in D'Alessandro and Rausch, 2008). Very few antigens deriving from *E. vogeli*, and none from *E.oligarthrus*, have been described in the literature, although *E. vogeli* can be maintained in vivo and in vitro (Ingold et al., 2001; Hemphill et al., 2003; rev. in Siles-Lucas and Hemphill, 2002). This scarcity of

antigenic data is also found in GenBank for the two species, in which only 51 protein entries for *E. vogeli* and 47 for *E. oligarthrus* are found (search date: 13/12/2015).

From these sequences, some of them could have potential in the diagnosis of polycystic echinococcosis, due to the findings of similar antigens with some diagnostic potential from *E. granulosus* and *E. multilocularis*. Among them, and from *E. vogeli*, it was described the partial sequences of the cytosolic MDH (Badaraco et al., 2008), AgB1 and AgB4 (Frosch et al., 1994; Haag et al., 2006) and AgB2 (unpublished, GenBank nb. AY324078), and from *E. oligarthrus* the partial sequences of AgB1, AgB2 and AgB5 (Haag et al., 2006).

From whole tissues of this parasite, Gottstein et al. (1995) described both a crude extract and the Ev2 antigen, an *E. vogeli* antigenic fraction purified from the metacestode extract by immunosorption. Extracts were obtained from whole cysts isolated from the peritoneum of infected jirds. For removing antigenic components shared by *E. vogeli* and *granulosus* and obtain the Ev2 fraction, the total *E. vogeli* extract was subjected to immunosorption with a hyperimmune serum raised against *E. granulosus* HF in rabbit. Both extracts were used in immunoblot and the ELISA (Gottstein et al., 1995).

3. IMMUNOLOGICAL DIAGNOSIS AND FOLLOWUP OF CYSTIC, ALVEOLAR AND POLYCYSTIC ECHINOCOCCOSIS IN HUMAN PATIENTS AND INFECTED ANIMALS

In the following sections, current information on the detection of antibodies, antigens, lymphoproliferation and cytokines in both CE and AE is presented. A summary of the usefulness, drawbacks and outlook of the different laboratory diagnosis options, including parasite detection, is shown in Box 1.

3.1 Detection of antibodies

3.1.1 In cystic echinococcosis patients and infected animals

Diagnosis and followup of CE patients is first approached with imaging techniques. The WHO-IWGE published a standardized ultrasonography (US)-based classification of stage-specific cystic images with detailed cyst status as follows: active (CE1, CE2), transitional (CE3a and CE3b) and inactive (CE4 and CE5) (Brunetti et al., 2010). This classification helps in the clinical management of human CE, since patients should be ideally treated

Box 1 Laboratory Diagnosis of Cystic Echinococcosis (CE) and Alveolar Echinococcosis (AE): Usefulness (U), Drawbacks (D) and Outlook (O)

Detection of Antibodies

U. The most frequently used test, EgHF-ELISA for the detection of IgG in serum of CE and AE patients, shows an acceptable sensitivity. Combined Em2 and Em18-ELISA systems as e.g., represented by the commercial Em2^{plus}-ELISA show a satisfactory sensitivity and specificity for detection of IgG in serum of AE patients.

D. The EgHF-ELISA IgG shows false negative and numerous false positive results, and it is useless for followup due to long persistence of anti EgHF IgG antibodies in CE and AE patients, regardless treatment outcome. Em18-ELISA IgG is suitable for monitoring treatment efficacy in AE patients. Confirmation of ELISA-based CE and AE serodiagnosis by immunoblot analysis is essential.

O. Alternative reagents (recombinant antigens, synthetic peptides—see Table 1) should be validated with extended panels of patients with known clinical variables (cyst stage, number, size and location; treatments), since they influence the presence and/or availability of detectable specific antibodies. A cohort including a minimum number of patients under each treatment modality (Brunetti et al., 2010) should be used to evaluate alternative reagents for followup of treated patients. For this validation, homogeneous technical approaches (antigen obtainment, type of antibody to be detected, technique, cut-off value and statistical analysis of the test results) should be implemented in a multicentre approach.

Detection of Antigens

U. Detection of circulating antigens could be of use to detect patients showing false negative results in antibody detection tests and for the followup of treated patients.

D. Cost and time-consuming techniques for the release from the antigen–antibody complex and for the concentration of antigens have to be applied before the detection.

O. The development of a commercial kit allowing the automatized and standardized release and concentration of antigens in a specific biological sample (serum, urine or saliva), and containing a defined antibody against antigens of *Echinococcus granulosus*, is desirable. Validation of this kit should be performed with an extended number of CE patients and with samples from patients with other parasitic diseases. While circulating antigens potentially suitable as diagnostic markers for CE have been described, the search for

Box 1 Laboratory Diagnosis of Cystic Echinococcosis (CE) and Alveolar Echinococcosis (AE): Usefulness (U), Drawbacks (D) and Outlook (O) (*cont'd*)

such circulating antigen markers suitable for diagnosis of AE should be intensified.

Detection of Cytokines

U. Detection of cytokines in serum by ELISA could be useful for the diagnosis and followup of both CE and AE patients.
D. Similar problems to those for antibody detection (false negative and unspecificity) are found. Additionally, seronegative patients are also negative in cytokine tests, thus cytokine detection is not more useful than antibody detection.
O. The decline of specific cytokine levels occurs faster than the decline of antibodies in treated and cured CE patients, thus usefulness of some cytokines for the followup of CE patients should be further validated. Developmental work on cytokine assays for followup of AE patients should be intensified.

Detection of the Parasite (Microscopy, Antigen Detection, Nucleic Acids Detection)

U. Protoscoleces and parasite material (antigens and DNA) can be specifically detected by microscopy and immunological/polymerase-chain-reaction-based techniques (see Sections 3.2. and 5).
D. Invasive techniques have to be applied to collect the material inside the cyst with the risk of spillage and secondary CE or metastasis formation causing secondary AE.
O. Detection of the parasite or its derivatives can be performed in patients subjected to interventional strategies (surgery or aspiration).

in relation with the cyst stage. It should be mentioned that some concerns about the cyst activity reflected in this classification have arisen in the last few years, especially regarding activity of CE3b and CE4 cysts. Cyst morphology and parasite viability are not always matching with this classification, as shown through studies of metabolite profiles in different cystic stages (Hosch et al., 2008). High concentrations of metabolites usually found in viable cysts were frequently found in CE3b cyst type and occasionally in CE4 cysts, suggesting that at least the transitional status of CE3b cysts should

be revised as active (Hosch et al., 2008). Similarly, antibody responses against CE4 cysts are variable depending on the treatment given to the patient prior to reaching the CE4 stage, e.g., antibody levels are higher in patients with CE4 cysts that have evolved from transitional stages after drug treatment than in patients reaching the CE4 stage without treatment (Sánchez-Ovejero et al., 2016).

E. granulosus cysts induce a strong antibody response in most patients, triggering different isotypes (IgG, IgM, IgA and IgE), although the intensity and specificity of the response depend on several factors. The first antibodies appear few weeks after infection against oncosphere antigens. Subsequently, antibodies are developed against the laminar layer and later against the cyst fluid and protoscoleces, if present.

The main serological methods used for human CE diagnosis and followup are based on the detection of specific IgG antibodies. The most widely used antigen for the detection of specific IgG antibodies is the HF. This antigen mixture is currently used in several techniques such as the ELISA, the indirect haemagglutination test (IHA) and the immunoblotting (IB). The immunoprecipitation agar technique, which was used for the detection of arc5, is nowadays rarely used due to several disadvantages (see Section 3.1.2). Both the ELISA and the IHA are usually the first line tests for CE patients, while the IB is used as confirmatory test. In this context, a number of drawbacks have been detected, including low sensitivity and specificity and a poor prognostic value for followup due to the long-lasting persistence of antibodies against HF (Barnes et al., 2012).

A number of recent studies that used the IgG-ELISA against HF antigens for CE diagnosis reported variable sensitivity ranging from 64.8% to 100% (rev. in Zhang and McManus, 2006; Carmena et al., 2006; Sarkari and Rezaei, 2015; Manzano-Roman et al., 2015). False negative results depend on several factors described by the different authors (rev. in Manzano-Roman et al., 2015; Lissandrin et al., 2016), including early (CE1) and inactive (CE4 and CE5) cyst stages (Schweiger et al., 2012; Wang et al., 2013; Tamarozzi et al., 2013), cyst location other than the liver (Akisu et al., 2006; Kilimciôglu et al., 2013), serum collection before treatment (Hernández-González et al., 2012; Tamarozzi et al., 2013), single and small cysts (Hernández-González et al., 2012) and HF antigenic source variability (Rahimi et al., 2011). Parasite genotype is an additional source of potential false negative results in serological tests with HF. The G1 and G2 genotypes from Europe, as contrasted to those from China, contain high quantities of antigen B2 in HF (Jiang et al., 2012a,b). Concerns about antigenic variability

of the HF among different parasite genotypes, as mentioned in Section 2.1 of this chapter, has been raised by several authors, leading to different diagnostic performance of a specific HF source for the detection of antibodies against different parasite genotypes (rev. in Manzano-Román et al., 2015). Other sources of antigenic variability are related with cyst stage, as showed by recent proteomic and immunoproteomic studies showing that CE1 and CE2 cyst stages differ in the expression of their immunodominant antigens (antigen B and antigen 5). Antigen 5 is predominant and recognized by antibodies from patients with early (CE1) and inactive (CE5) cyst stages, while antigen B is most scarce in CE1 cyst stage and mainly detected by antibodies from patients with CE2 and CE3 cyst stages (Ahn et al., 2015a).

A second problem is the percentage of false positive results. IgG-ELISA based on the use of HF as antigen gives rise to variable false positive results in healthy donors from different geographical areas (e.g., specificity of 53.8% in Iranian donors, of 87.5% in Indian donors and of 100% in Italian donors) (Mohammadzadeh et al., 2012; Zhang et al., 2012; Tamarozzi et al., 2013). Cross-reactivity of antibodies against HF is found in patients with other parasitic and nonparasitic diseases, among them alveolar echinococcosis (AE), cysticercosis (rev. in Eckert and Deplazes, 2004; Carmena et al., 2006; Manzano-Roman et al., 2015), clonorchiasis (Jin et al., 2013), fasciolosis and schistosomiasis (Tawfeek et al., 2011), ascariasis, amoebiasis and malignancy (Chirag et al., 2015), taeniasis, paragonimiasis, hookworms, gnathostomiasis, strongyloidosis, trichinellosis, capillariasis and trichuriasis (Dekumyoy et al., 2005). Especially high is the cross-reactivity of HF with AE patients (>50%; Hernández-González et al., 2012). An effort should be done to define the extent of cross-reactivity of the HF for additional parasitic diseases commonly found in endemic areas such as toxoplasmosis and others. The accurate definition of the unspecific reactivity of the HF for both healthy donors and patients with other diseases is still needed to have a precise view of the usefulness of serological tests based on the use of this antigenic source in specific settings.

As mentioned, the HF has also shown weaknesses when used as antigenic source for the followup of antibodies in CE patients under clinical management. During the followup, HF ELISA-IgG is difficult to interpret due to antibody levels fluctuations not attributable to changes due to treatment (Galitza et al., 2006), and anti HF IgG antibody reactivity may remain high many years after successful cyst removal (Lawn et al., 2004). In this sense, the detection of antibodies other than IgG has shown better results than the detection of total IgG in relation with patients' followup (Tenguria

and Naik, 2014), although this is still a question of debate (Galitza et al., 2006). IgE and IgM antibodies have shown to be better markers than IgG after chemotherapy and surgery (Zarzosa et al., 1999). It has been also shown that both IgG2 and IgG4 against HF could be related with cyst stages, disease evolution and relapses (Celik et al., 2009; Benabid et al., 2013). Remarkably, it is known that the subisotype responses against CE1, CE2 and CE3 cyst stages are mainly of the IgG4 subisotype, while IgG1, IgG2 and IgG3 responses predominate against CE4 and CE5 cysts (Carmena et al., 2006; Moro and Schantz, 2009). Although still an open question, this antibody shift could be related with the stimulation of antibody responses driven by the LL of the cysts in active and transitional stages, resulting in the triggering of TregCD8^{+}FoxP3^{+} cells and thus IgG4 antibodies, and the loss of dominance of the LL components in the stimulation of humoural responses in patients with inactive cysts. The level of antibody isotypes or subisotypes different from total IgG, nevertheless, is more frequently under detection in CE patients than IgG (Marinova et al., 2011; Tawfeek et al., 2011; Cappello et al., 2013), limiting their usefulness in CE serodiagnosis.

In summary, the antibody response against the HF is variable both qualitatively and quantitatively in different patients and in the same patient at different times post infection. This variability depends on several clinical variables, including cyst stage, number of cysts, cyst size and location, treatment followed by each patient and parasite genotype, among others (rev. in Manzano-Roman et al., 2015; Lissandrin et al., 2016). These pitfalls, together with those found in the applicability of this antigen mixture in the followup of CE patients, lead clinicians to consider the detection of antibodies against HF in ELISA as an approach with doubtful benefit for the clinical management of CE.

Alternative extracts from other compartments of the cyst apart from HF have been evaluated for their performance in serological tests. The total somatic extract of protoscoleces has been assayed by several authors, resulting in variable sensitivity ranging from 69.4% to 96.9% (Chamekh et al., 1990; Ersfeld et al., 1997; Rafiei and Craig, 2002; rev. in Carmena et al., 2006; Zhang and McManus, 2006; Swarna and Parija, 2008; Feng et al., 2010; Schweiger et al., 2012; Fotoohi et al., 2013; Chen et al., 2015; rev. in Manzano-Roman et al., 2015; Sarkai and Rezaei, 2015). In general, specificity has been worse for the extract of protoscoleces, compared with HF. Other extracts have been assayed less frequently, including somatic extracts of adult worms, of protoscoleces tegument and of cyst wall, with sensitivity ranging from 81.3% to 96.7% with false negative results attributed to similar

reasons as that for HF (Chamekh et al., 1990; Ersfeld et al., 1997; Schweiger et al., 2012; Mohamed et al., 2014). A common problem for all these extracts is their cross-reactivity and their heterogeneity, very similar to that found for HF.

A number of purified native antigens have been tested for the detection of antibodies in CE patients, mainly representing AgB and Ag5. Unfortunately, similar drawbacks than for HF are encountered when purified antigens from this source are used, including false positive results probably due to the presence of cross-reacting carbohydrate moieties in purified native antigens, false negative results attributed to clinical variables already pointed out for HF, and variability in sensitivity and specificity using the same purified antigen due to lack of standardization of purification methods, among others, both in ELISA and immunoblot (rev. in Carmena et al., 2006 and Manzano-Roman et al., 2015). Similarly, the use of antigens purified from different sources of HF (e.g., AgB) result in variable sensitivity in the same cohort of patients (from 82.1% to 96.9%; Mohammadzadeh et al., 2012), showing an intrinsic variability of native antigens. Ranges of specificity and sensitivity for the different native antigens can be found in Table 1.

With the objective of developing more sensitive and specific tests, several recombinant antigens have been also described and tested. These are mainly represented by different subunits of AgB and Ag5, and some additional molecules from HF or protoscoleces, detailed in Table 1. Findings of variable sensitivity and specificity are common for all the recombinant antigens tested more than once by different authors in different patients' cohorts (Table 1). False negative results have been attributed to clinical variables similar to those reported for HF, including cyst number, size, location and stage, complications and treatment before serum collection (rev. in Manzano-Román et al., 2015). The parasite genotype has been also pointed out as a potential cause of false positive serology in CE patients against defined antigens (rev. in Manzano-Román et al., 2015), although the ascertainment of such variability has not been deeply studied for each of the antigens. In this regard, Boubaker et al. (2014) compared the reactivity of antibodies from CE patients infected with the G1 and G6 *E. granulosus* genotypes against the recombinant P29. Minor amino acid variations were found for the antigen in the two genotypes. Accordingly, reactivity of patients infected with G6 or G1 cysts were similar against the recombinant P29 obtained from the G1 genotype (Boubaker et al., 2014).

Most of the available studies applying recombinant antigens or synthetic peptides for the diagnosis of CE patients are usually underpowered clinical studies, mainly due to the small number of tested samples and the lack of information on clinical variables of the patients under study. To date, few intercentre studies have been undertaken. This is a major need, since antigens preliminary representing the same reactive but tested with different protocols and serum collections give rise to variable results, precluding the clear definition of their potential use in the management of CE patients. One example of this approach was published by Lorenzo et al. (2005), who performed a double-blind analysis by a network of six South American laboratories. Testing of HF, purified AgB, recombinant AgB1 and AgB2 and MDH, and the peptide p176 in IgG-ELISA was done, finding the highest sensitivity for HF, purified AgB and recombinant AgB1 (around 81%), and the best specificity for the recombinant antigens and the synthetic peptide. The use of a common approach for the definition of cutoff values for each antigen seems also to be very important for the evaluation of antigens. This study used a common Receiver Operating Charateristic (ROC) curve analysis for this evaluation, finding an almost complete agreement in the interlaboratory classification of positive and negative sera. Strikingly, this approach resulted in the lack of significant differences among different HF batches, thus the HF emerging as a 'homogeneous' antigen in this setting. Also intriguingly, the worst results in the interlaboratory repeatability were attributed to the p176 synthetic peptide (Lorenzo et al., 2005).

In this approach and in the majority of the test done with recombinant antigens, around one-fifth of the CE patients' sera gave rise to false negative results (Lorenzo et al., 2005, Table 1). Complementarity among the antigens tested by Lorenzo et al. (2005) was also assayed, but the percentage of false negative sera was found to be the same as for the individual antigens. This was attributed to the lack of a measurable antibody response in false negative patients potentially related with the disease stage. Similarly, combination of several peptides in the same tests did not result in higher sensitivity than the sensitivity of single peptides (List et al., 2010). Attempts to enhance the reactivity of defined recombinant antigens by cloning and expressing tandem repeated sequences was also not fully successful in avoiding false negative reactions (Hernandez-Gonzalez et al., 2012).

When recombinant antigens with a preliminary good performance in clinical settings have been applied for the detection of antibodies in screened people showing images compatible with CE lesions, results have been discouraging. An example of this can be found for the antigen EpC1 when

used in screened population in Peru (Gavidia et al., 2008), showing the limited performance of available serologic assays in the field. The potential of combining several recombinant antigens to enhance the performance of serological tests, either in clinical settings or in the field should be further explored.

Very few recombinant antigens have been characterized regarding their usefulness in followup of CE patients for the three main modalities of clinical management (surgery/aspiration, drug treatment and watch and wait; Brunetti et al., 2010). For followup after drug treatment and in some instances after treatment based in aspiration techniques, it has been mentioned that some antigens could be mainly expressed or are mainly detected by specific antibodies when defined cyst stages are found in the patients. This shows that serology against defined recombinant antigens could be potentially useful for the followup of CE patients in which treatment results in changes from active or transitional cysts to inactive cysts, e.g., against Ag5, AgB2, HSP20 and AgB1 (Li et al., 2004, 2010; Vacirca et al., 2011). For instance, the recombinant AgB2 in ELISA showed a sensitivity of 74%, 96%, 90%, and 56% in patients with CE1, CE2, CE3, and CE4/CE5 cyst stages, respectively (Li et al., 2010). Ag5 also seems to induce antibodies mainly when CE1 and CE5 cysts are found (Ahn et al., 2015a), although this has not been demonstrated with the recombinant Ag5, thus the usefulness of this antigen for the detection of early active and late inactive cyst lesions should be further evaluated.

A good correlation of loss of specific antibodies after successful surgical treatment in patients has been reported against the recombinant AgB, AgB2t, AgP29, and HSP20 (Li et al., 2004; Hernández-González et al., 2008; Ben Nouir et al., 2009; Vacirca et al., 2011). Nevertheless, the percentage of CE patients with detectable levels of antibodies against some of the above-mentioned antigens is limited (e.g., against AgP29; Ben Nouir et al., 2009). The banding pattern of HF recognized in immunoblot by CE patients in followup could also be useful in their clinical management, since these changes are found depending on the cyst stage and in cured patients (Mariconti et al., 2014), but no additional antigen has been defined so far based on these changes. Similarly, the shift of antibody isotypes and subisotypes along cyst evolution could be useful for followup, including the correlation of IgG4 levels with cyst activity and the decline of antibodies of isotypes different from IgG in cured patients, especially of IgE and IgM (Lawn et al., 2004; Celik et al., 2009), although antibodies different from

IgG are lacking or under detectable levels in a percentage of CE patients (rev. in Manzano-Roman et al., 2015).

The majority of the information published on the use of different recombinant antigens and their performance in the serodiagnosis of CE patients has been generated in the laboratory and usually has not been implemented in the clinical practice. The mention to commercial kits and new easy-to-use devices in the following paragraphs will give an overview about the alternatives that clinicians could find now or in the near future for the diagnosis of CE patients.

Several kits mainly based on ELISA, IHA and immunochromatography, containing either crude HF or semipurified fractions of this antigenic mixture are commercially available, although some of them do not specify the antigenic source present in the kit. Many of them have been validated with a limited number of sera from patients of unknown clinical characteristics, when detailed. The results of extended validations and comparison with gold standard(s) have been published only for few of them. Among them, the IHA test Fumouze (http://www.fumouze.com/produit/hydatidose-fumouze/) has been tested by several authors, with variable results in sensitivity (from 34.9% to 88%; Auer et al., 2009; Bilge et al., 2009; Liance et al., 2000; van Doorn et al., 2007; Hernandez-Gonzalez et al., 2012) and specificity (e.g., from 44% to 70% for AE patients; Liance et al., 2000; Auer et al., 2009; Hernandez-Gonzalez et al., 2012). Similarly, several ELISA commercial kits have been tested and compared with in-house ELISAs, showing variable false negative and false positive results for the commercial kits (e.g., Liance et al., 2000; Paul and Stefaniak, 2001; Auer et al., 2009). Commercial kits based on the immunoblot technique are also available, e.g., the *Echinococcus* Western Blot IgG (LDBIO Diagnostics). This kit contains the whole larval extract of *E. multilocularis*. Interpretation on the sensitivity and specificity is done depending on the resulting banding pattern, although this is somehow difficult to interpret, since CE patients should recognize a band at 7 kDa and/or diffuse band of around 18 kDa, and AE patients should specifically recognize a band at around 28 kDa, among others (Liance et al., 2000). Western blot results in better sensitivity than the ELISA and the IHA techniques (e.g., Liance et al., 2000).

In the last few years, attempts for the development of a sensitive and easy-to-use tool have been done. These are mainly based in immunochromatographic tests (ICT) and dot immunogold filtration assay (DIGFA), from which some of them have reached the market. ICT is advantageous in comparison with ELISA, Western blot and IHA in several of its

characteristics, including short assay time, no need of specialized personnel and easy to interpret results. They are usually more economic than other techniques and do not need a cold chain to be transported or stored. These characteristics make ICT-based tests very valuable in low income settings. For ICT, variability in the antigens used in each test can give rise to different sensitivity. This is illustrated in three recent publications using a commercial ICT containing a semipurified fraction of HF Ag5 (Virapid Hydatidosis, Vircell), and two in-house ICT containing crude HF and the recombinant AgB1, respectively (Wang et al., 2013; Santivañez et al., 2015; Tamer et al., 2015). The highest sensitivity was obtained with the commercial ICT (96.8%; Tamer et al., 2015), followed by the ICT containing crude HF (91%; Wang et al., 2013) and the ICT containing the recombinant AgB1 (78%; Santivañez et al., 2015). The ICT with crude HF showed dissimilar sensitivity for cysts at different stages (93.4% for active and transitional cysts and 42.8% for inactive cysts) showing the potential of this test to discriminate between active-transitional and inactive cysts in CE patients (Wang et al., 2013). This assay has been recently commercialized (EUROLINE-WB, Euroimmun) and an extended review on its performance is detailed in Section 3.1.2. Specificity of the three ICTs was around 90%. DIGFA has been also used for the diagnosis of CE patients. Recently, a DIGFA test that allows combination of multiple antigens was developed by Feng et al. (2010). The advantage of combining several antigens in the same device is to give the chance of evaluating the response against highly sensitive and moderately specific antigens together with less sensitive but more specific antigens at the same time. This DIGFA test contains four native antigens standing separately: crude HF, native purified AgB, protoscoleces extract and the *E. multilocularis* Em2 antigen. Its overall sensitivity was 80.7%, with a specificity of 93.4% against AgB (Feng et al., 2010). These new ICT and DIGFA devices, although promising, should reach an agreement on their standardization to reach the market. Undoubtedly, the most sensitive ELISAs or ICTs containing HF or semipurified fractions of this crude antigen could be used as primary test in a routine laboratory for the diagnosis of CE. Nevertheless, a second, more specific test containing recombinant antigens or peptides should be performed to corroborate the diagnosis.

A flow-chart on how to approach the laboratory diagnosis of CE and AE is shown in Fig. 4.

It is worth mentioning the recent developments for the detection of specific antibodies in body fluids different from serum and generally less difficult

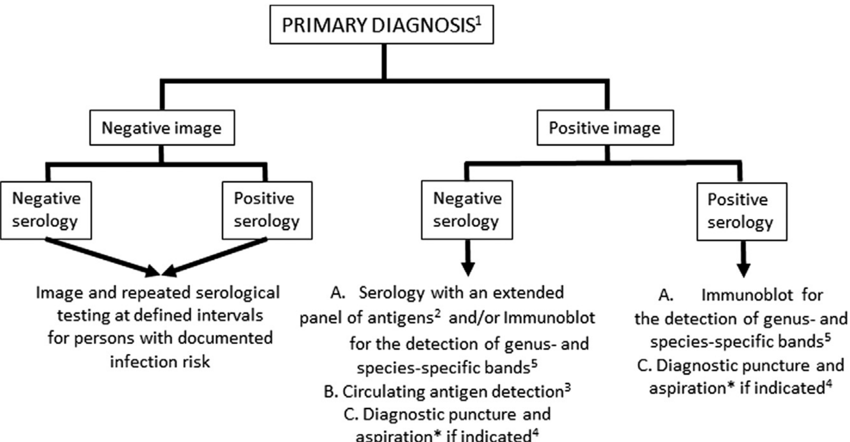


Figure 4 Schematic approach for the laboratory diagnosis of cystic echinococcosis (CE) and alveolar echinococcosis (AE) in human patients. [1]For suspected clinical cases, not for findings in population screening, perform the imaging procedure, complemented by serology; for serology, IgG-enzyme linked immunosorbent assay (ELISA) against hydatid fluid for CE patients and Em2plus ELISA (Bordier Affinity Products, Crissier, Switzerland) for AE patients is usually performed. [2]IgG-ELISA against purified antigen 5 and/or recombinant B antigen for CE patients and e.g., recombinant EM13 for AE patients. [3]Treatment of serum with 3% polyethylene glycol and detection of antigens with peroxidase conjugated hyperimmune human hydatid IgG (Gottstein, 1984; Craig, 1986) for CE patients (not described for AE patients). [4]Material can be used for microscopy to detect protoscoleces or for specific multiplex PCR (Trachsel et al., 2007; Boubaker et al., 2013). [5]With Western Blot IgG (LDBIO Diagnostics Lyon, France) or EUROLINE-WB IgG (Euroimmunn, Lübeck, Germany). *Fine-needle aspiration biopsy for AE patients. *Adapted from Siles-Lucas, M.M., Gottstein, B.B., 2001. Molecular tools for the diagnosis of cystic and alveolar echinococcosis. Trop. Med. Int. Health 6, 463–475.*

to collect and handle. Urine from CE patients have been tested by ELISA against HF for the detection of IgG, IgG1, IgG4, IgE and IgM (Sunita et al., 2007; Chirag et al., 2015), showing a sensitivity similar to that found in ELISA for total IgG in serum (from 80.5% to 84%) and a higher specificity. The use of saliva resulted in lower specificity in IgG-ELISA against HF (56%; Sunita et al., 2007). Thus, urine may be used as an alternative to serum samples by virtue of its noninvasive, easy collection and similar sensitivity and specificity.

Ideally, an easy-to-use test containing several recombinant antigens placed individually in the device could act both as a primary test and as a specific confirmatory test in clinical settings, and could provide better information on the outcome of CE patients under the different management modalities. Nevertheless, the potential use of preliminary promising

recombinant antigens and synthetic peptides for the management of CE patients is still hampered by the lack of sound criteria that may or may not justify their utilization, either alone or in combination. In this regard, the agreement on the obtainment of defined and standardized antigenic preparations and their validation in a multicentre approach with an ample panel of serum samples from patients with detailed clinical history in needed. The definition of the antibody isotype(s) to be detected and the means for this detection is also of importance. Latest, an agreement on the methods used to calculate the cutoff value for the tests under validation should be reached. Recently, HERACLES project (http://www.heracles-fp7.eu/index.html) funded by the European Commission, was launched and among their activities two web-based databases have been developed to prospectively and retrospectively register CE patients and their related clinical data (European Register of Cystic Echinococcosis −ERCE, http://www.heracles-fp7.eu/erce.html and CYSTRACK, http://cystrack.irnasa.csic.es/login). Additionally, a biobank for the storage of samples from CE patients, including serum samples, have been linked with these two databases. Hopefully, the availability of these tools will help to define the usefulness of different antigenic preparations in the diagnosis of CE patients, including if the reasons of false-positive reactions can be attributed to the lack of measurable specific antibodies in defined clinical settings (e.g., small and single cysts) or, on the contrary, only some antigens will be useful to define specific cyst stages independently of other clinical variables like cyst size, number and location, parasite genotype and others.

The detection of specific antibodies in animals infected with *E. granulosus* cysts show similar problems than those found for CE in humans. The poor results found in sheep with CE of the tests for the detection of specific antibodies evaluated until now are comparable to those found in human population screening, giving rise to very low sensitivity and specificity. Attempts to study the evolution of antibody responses in experimentally infected sheep have shown that the course of antibody responses is similar to that found in patients, showing first antioncosphere antibodies, followed by antibodies against the HF, including antibodies against AgB and Ag5, and the protoscoleces (rev. in McManus, 2014). The results of serological tests for CE in sheep have been generally disappointing and often contradictory. Noteworthy, these poor results are also found with serological tests for the detection of other tapeworms in animals (rev. in McManus, 2014).

As mentioned, the serological diagnosis of CE in animals has two main problems: cross-reactivity with other cestodes and false positive with healthy

animals, and low sensitivity due to low responses against cyst antigens (rev. in McManus, 2014; Craig et al., 2015). A standardized serological method for the individual diagnosis of animal CE is not available to date, and those methods available are limited by the use of crude native antigens such as the HF, thus very heterogeneous and difficult to compare among different laboratories. An example of this variability can be found in two publications. The first is a study performed in New Zealand on the detection of antibodies in sheep using a protosocoleces extract, the purified AgB from HF and the vaccine antigen EG95 (Kittelberger et al., 2002). Tested animals were both naturally and experimentally infected sheep, and the highest sensitivity was obtained with the protosocoleces extract (62.7%), with a good specificity (around 90%). Due to its limited sensitivity, this test could be applied to detect herd infection, but not in individual animals. The low reactivity of AgB found in this study is not consistent with previous results of other authors, which may reflect the heterogeneity of AgB depending on the HF source, parasite genotype, antigenic isoform, purification method, etc. The second example published by Gatti et al. (2007) uses the crude HF and two purified fractions of HF, testing these antigens in naturally infected sheep. The HF gave around 90% sensitivity in sheep older than 7 months and the specificity was high. In general, information about the use of serology in animals with CE is scarce and heterogeneous, precluding the extraction of sound conclusions about the usefulness of this approach for the detection of infected animals. The available publications on this subject lack information on variables that could be important for the definition of the usefulness of the applied serological test, including cyst number, location, stage and others. Studies on the proteomic (antigenic) composition of sheep cysts done recently (Ahn et al., 2015a) could help in the definition of the best a priori candidates for the detection of specific antibodies in animals with CE.

3.1.2 In alveolar echinococcosis patients and infected animals

Initial assessment of a symptomatic AE in a human patient normally consists of a tentative diagnosis based on information from anamnesis and subsequent physical imaging demonstrating the vesicular conglomerates from the *E. multilocularis* metacestodes inside the liver tissue (e.g., rev. in Siles-Lucas and Gottstein, 2001; Moro and Schantz, 2009). Verification of this initial diagnosis is routinely performed by serology that nowadays provides a set of reliable assay systems that allows sensitive detection of *Echinococcus*-specific antibodies in sera from AE patients (Fig. 4). However, serology is even more

useful for early diagnosis of AE particularly in a situation before the disease becomes manifest and at a time point when radical surgical resection of the infected liver tissue is still a realistic option (e.g., rev. in Siles-Lucas and Gottstein, 2001).

As described above, major efforts have been made over many years to achieve a reliable immunodiagnosis of CE in human and animal patients (see Section 3.1.1). In AE, respective developmental work has been more limited because more than 10 years ago some pioneer studies were already quite successful in that they provided at least a small set of native and recombinant *E. multilocularis* antigens allowing highly sensitive and specific diagnosis of the disease (e.g., rev. in Siles-Lucas and Gottstein, 2001; Carmena et al., 2007). Anyway, the identification of some new antigens and the development of novel concepts for serological analyses in recent years contributed to a substantial progress in immunodiagnosis of AE particular as far as posttreatment serological monitoring of AE patients is concerned.

For long time, crude preparations of EgHF from *E. granulosus* (genotype G1) cysts isolated from sheep was the only antigen available for immunodiagnosis of AE in humans. EgHF was initially used as antigenic reagent for an immunoelectrophoresis assay (see above) where serological positivity revealed an antibody-Ag5 immune complex resulting in a visibly accurate precipitation band termed arc5 (e.g., rev. in McManus, 2014). This assay was actually developed for immunodiagnosis of CE both in humans and domestic animals (e.g., rev. in McManus, 2014). However, in the following case, it was also evaluated regarding its immunodiagnostic application as far as human AE is concerned. As for example reported by Schantz et al. (1983), Ag5-based immunopreciptation detected serum anti *E. multilocularis* antibodies in human patients at a sensitivity of 69%. However, this finding had to be put into perspective of a previous study, where Ag5 exhibited strong cross-reactivity to sera representing cases with other helminth infections (Varela-Díaz et al., 1978). Accordingly, the arc5 assay may have a certain value as a serological screening test but it does not allow *Echinococcus*-genus or even -species differentiation on the immunodiagnostic level. Regarding the sensitivity, the arc5 assay did not even reach the level of the IHA test that in a parallel analysis provided a positive result for 16 out of 17 AE patients (Schantz et al., 1983).

Compared to the arc5 test, significantly improved diagnostic operating characteristics of a classical screening test in AE (and CE) were attributed to ELISAs including EgHF, or EmVF, as antigenic reagents. For example, in a large-scale study performed with sera from patients with AE, CE, and

various other parasitic diseases, the EgHF and EmVF-ELISAs revealed high diagnostic sensitivities (AE: 81% and 89%, CE: 91% and 95%) but rather low specificities (58% and 72%) with respect to diagnosis of non *Echinococcus* parasitic infections (Müller et al., 2007). Further complications in these ELISAs were caused by false positive results in serology from patients affected by different types of cancer malignancies (Pfister et al., 1999; Müller et al., 2007). Conversely, it was found that most (7 of 8) or even all (8 of 8) investigated cases of aborted AE carrying calcified lesions scored negative in the EmVF- or EgHF-ELISA, respectively.

Apart from EgHF and EmVF, a few other native antigen fractions were tested regarding their diagnostic suitability in AE (Schweiger et al., 2012). These were antigen P isolated from purified protoscolices and an Em2-deprived subfraction of this antigen, named P1. In ELISA, both antigens exhibited similar diagnostic operating characteristics as a crude *E. multilocularis* metacestode antigen, named EmC. All three antigens turned out to be suitable for screening tests in AE (and CE) but respective assays did not even reach the specificity levels of EmVF and EgHF as far as differential diagnosis between AE (and CE) and other parasitic infections are concerned.

Several years ago, a major progress in immunodiagnosis of AE was achieved by the isolation of the affinity-purified, native metacestode antigen Em2 (Gottstein et al., 1983, 1993: Müller et al., 2007). This antigen represents a carbohydrate complex located in the LL that has been purified by affinity chromatography using either a polyclonal antibody against EgHF (Gottstein et al., 1983) or Em2-specific monoclonal antibody G11 for immunoabsorption of the antigenic components (the latter named antigen Em2G11; Deplazes and Gottstein, 1991). Of similar relevance in this respect was the subsequent identification of a series of near-identical recombinant antigens, namely EmII/3 (Vogel et al., 1988), EmII/3−10 (Müller et al., 1989; Felleisen and Gottstein, 1993), EM10 (Frosch et al., 1991; Helbig et al., 1993), EM4 (Hemmings and McManus, 1989, 1991), and Em18 (Ito et al., 2002; Xiao et al., 2003; Li et al., 2010) that altogether represent highly antigenic and overlapping subfragments from the 18 kDa EMR-protein. The application in ELISAs of Em2 and/or one of the recombinant 18 kDa antigens listed above basically allowed the discrimination between AE and CE as well as cysticercosis (caused by the metacestode of *Taenia solium*) and other diseases caused by various parasitic nematodes and trematodes (Müller et al., 2007). As exemplified by Gottstein et al. (1993), immunodiagnosis of human AE based on Em2-and EmII/3-10-ELISA provided excellent test results. Here, large-scale testing of the two antigens as a

cocktail using the commercial Em2^{plus}ELISA (Gottstein et al., 1993) resulted in an overall cumulative sensitivity of approximately 97% as compared to approximately 89% and 86% achieved individually for antigen Em2 and EmII/3–10, respectively (Gottstein et al., 1993). In this study, both antigens exhibited a high to moderate specificity revealing values of approximately 94% (Em2), 95% (EmII/3–10), or 74% (Em2^{plus}) in differential AE versus CE diagnosis and 100% (Em2), or 98% (II/3–10 and Em2^{plus}) regarding diagnosis of AE versus other parasite infections. In a subsequent study performed by the same group, parallel testing of the two antigens in ELISA demonstrated somewhat lower sensitivities in that 90% (Em2), 79% (II/3–10), and 97% (calculated cumulative sensitivity) of AE cases were detected (Müller et al., 2007). Here, ELISAs also provided high specificity values namely 95% for both antigens regarding differential diagnosis of AE versus CE and 95% (Em2) or 92% (II/3–10) when cases with other parasitic diseases were serologically analyzed. Moreover, in the case of the EmII/3–10 ELISA, a few sera (2 of 18) from patients with cancer malignancies exhibited a low unspecific reactivity to the antigen.

In a recent study, a comparative assessment of three ELISAs using Em2, Em18, and another recombinant *E. multilocularis* antigen, EmP29, for serodiagnosis of human AE was carried-out (Boubaker et al., personal communication). This study showed that EmP29 is a good candidate antigen in order to complement Em2- and Em18 ELISAs and probably also other serological assays currently applied in routine immunodiagnosis of human AE.

In order to evaluate an immunodiagnostic method alternative to serology, Itoh et al. (2013) assessed the Em18-ELISA regarding its capability to detect anti *E. multilocularis* antibodies in urine samples from human AE patients. Here, particularly in an attempt aimed at the detection of specific IgG4 in AE patients versus patients with non *Echinococcus* parasite infections a surprisingly high diagnostic sensitivity (78%) and specificity (100%) was obtained. Unfortunately, this study did not provide any information about the potential of the assay to discriminate between AE and CE. Nevertheless, respective data indicated that ELISA-based testing of urine instead of serum samples represents an interesting concept to perform immunodiagnosis of echinococcosis thus avoiding the invasive procedure of blood sampling as e.g., desirable for large-scale epidemiological investigations in endemic regions.

In the early 1990s, ELISA-based serology in AE was complemented by various other assays in the same format (e.g., rev. in Siles-Lucas and Gottstein, 2001; Carmena et al., 2007). One of these ELISAs included

recombinant EM13 as diagnostic antigen (Frosch et al., 1993). In agreement with the observation that this antigen is expressed in *E. multilocularis* but not in *E. granulosus* metacestodes, recombinant EM13 demonstrated 100% specificity for diagnosis of human AE. Since the sensitivity of this assay turned out to be at least satisfactory (82%) it is surprising that this antigen was apparently not further evaluated regarding its applicability in differential diagnosis of AE versus CE. Subsequently, another ELISA utilizing a partially purified Em18/16-enriched fraction (PP-Em18/16) prepared from *E. multilocularis* protoscolices extracts by isoelectric focussing was evaluated for specific immunodiagnosis of AE (Ito et al., 1997). Although the authors did not report on overall serological sensitivities and specificities regarding the entire panel of patient sera included in the study, this assay seemed to allow a slightly better AE versus CE discrimination as the commercial Em2^{plus}ELISA tested in parallel.

In another study, the antigenicity of recombinant EmAgB1 (rEmAgB1) was serologically tested and compared with that of the recombinant *E. granulosus* analogue (rEgAgB1) (Mamuti et al., 2004). Respective Western blots and ELISAs were performed with sera from patients with confirmed CE or AE, respectively. Here, both antigens exhibited positive reactions with more than 80% of CE and approximately 40% of AE patients' sera with both antigens.

Although the ELISAs e.g., based on antigens Em2 and II/3—10 exhibit excellent diagnostic characteristics a lack of reactivity was observed in a small number of confirmed AE cases (Gottstein et al., 1993; Müller et al., 2007). Weak antibody reactivity is an occasional phenomenon in AE immunodiagnosis, particularly related to cases where the *E. multilocularis* infection was either recently acquired or has taken an abortive course (i.e., necrosis of the parasite in tissue). Furthermore, minor cross- or unspecific reactivity of some sera, particularly those from cancer patients, may hamper the correct interpretation of the results (e.g., Pfister et al., 1999; Poretti et al., 1999). Under these considerations, immunoblot analysis turned out to be well suited to complement ELISA-based immunodiagnosis of AE (and CE) (Poretti et al., 1999; Liance et al., 2000; Doiz et al., 2001; Furuya et al., 2004; Müller et al., 2007). One of the immunoblots previously described was based on the use of EgHF and relied on the detection of the 8 kDa subunit of antigen B (see above) and two other immunodiagnostically relevant antigens of 29 and 34 kDa (Poretti et al., 1999). In this study, all three antigens revealed to be genus-specific and allowed serological diagnosis of both AE and CE with an overall sensitivity of 91% and a specificity of 97% and

94% for 8 kDa subunit of antigen B and the 29/34 kDa antigens, respectively. At the same time, it became obvious that this immunoblot was not suited for a serological discrimination between AE and CE.

In a similar approach, Ito et al. (1999) assessed by IB the diagnostic characteristics of *E. granulosus* 8 kDa antigen B in combination with a native 18 kDa antigen (Em18) fraction previously enriched by isoelectric focussing from an extract of purified *E. multilocularis* protoscoleces. A large-scale serological evaluation of this dual immunoblot assay confirmed the genus-specificity of antigen B in that sera from AE and CE patients reacted at a frequency of 79% and 92%, respectively. Conversely, antigen Em18 turned out to detect AE cases with a high diagnostic sensitivity of 97% and at least a moderate specificity of 74% thus indicating the potential of the Em18 immunoblot to differentiate AE from CE. Furthermore, Liance et al. (2000) evaluated a commercial immunoblot kit referred to as *Echinococcus* Western Blot IgG (LDBIO Diagnostics Lyon, France) that utilizes crude protein extract from *E. multilocularis* metacestodes as antigen. In this assay system, *Echinococcus*-specific antibodies in patient sera reacted with a 7 kDa band and/or a diffuse 26—28 kDa band. The blot was able to detect AE and CE with diagnostic sensitivities of 96.7% and 98%, respectively. According to the instructions of the manufacturer, intermediate bands between the 7 kDa and 26—28 kDa bands allow thereafter the differentiation between AE and CE in more than two third of cases. Moreover, significant cross-reactivity was observed with sera from patients suffering from neurocysticercosis (7 of 20) or schistosomosis (3 of 18 sera).

In another immunoblot approach, Korkmaz et al. (2004) evaluated two *E. multilocularis* metacestode antigens, namely Em70 and Em90. Here, the respective large-scale evaluation revealed a surprisingly high sensitivity (100%) and specificity (99.1%) for the diagnosis of AE. However, these data as well as findings from most of the other serological studies described above have to be interpreted with caution. This is the case because respective investigations did not consider 'diagnostically critical' sera, particularly those originating from patients with abortive AE (see above). Such sera tend to remain negative if their reactivity to native or recombinant *E. multilocularis* antigens is tested. In this respect, a significant progress was achieved by the development of an immunoblot that utilizes an in vitro-produced EmVF antigen instead of native antigen normally prepared from alveolar cysts of experimentally infected rodents (Müller et al., 2007). This blot revealed an abundant immunoreactive band triplet of 20—22 kDa. In combination with the Em2-and EmII/3—10-ELISA

(Gottstein et al., 1993), this immunoblot method allowed diagnosis of both clinical and subclinical (including abortive) cases of AE with a maximal sensitivity of 100%. While the specificities regarding cases of both non *Echinococcus* parasite infections as well as cancer malignancies were also 100%, the specificity regarding CE was only 50% thus excluding the application of this immunoblot assay for discrimination of AE from CE.

In order to provide an improved tool for a reliable immunodiagnosis of, and a serological discrimination between human AE and CE, a combined membrane strip system composed of an immunoblot containing electrophoretically separated EmVF and three membrane chips coated with either recombinant 8 kDa AgB, Em18, or the as yet only marginally characterized recombinant *E. multilocularis* antigen Em95 (Wang et al., 2014), respectively. This assay recently commercialized as EUROLINE-WB (IgG) (Euroimmun, Lübeck, Germany) was extensively evaluated with sera from blood donors as well as precharacterized sera from patients with AE, CE, or various other parasitic diseases (Schönfeld et al., 2016). By using an automated algorithm for reading of the strips, a sensitivity of 93% and a specificity of 100% for diagnosis of echinococcosis versus other parasite infections were achieved. More important, however, was the finding that this assay was able to determine the respective *Echinococcus ssp.* in 81% of the positive results. Remarkable was also the observation that no serological cross-reactivity to any diagnostically relevant parasitic diseases including e.g., cysticercosis, schistosomiasis, filariasis, and amoebiasis was found. Conversely, unexpected cross-reactivity was obtained in patient sera that contained antibodies against *Ascaris lumbricoides* (4 of 11 sera) and *Anisakis simplex* (1 of 16 sera).

The EUROLINE-WB (IgG) assay certainly has to be considered as a substantial progress towards an optimal immunodiagnosis of AE and CE. Nevertheless, application of this assay and/or a combined use of different ELISAs and immunoblots described above do not allow an optimal discrimination between AE and CE in human patients. At the same time, false-positive results e.g., due to cross-reactive antibodies in patient sera still remains an important issue that may complicate the interpretation of AE and CE serology. Furthermore, production of native and recombinant antigens for immunodiagnosis of *Echinococcus* ssp. infections is time-consuming and hampered by batch-to-batch variation. As already outlined above, such limitations may be overcome by profiting from the possibility to chemically synthesize large amounts of pure antigenic peptides under standardized conditions. In this context, a pioneer study was employed by List et al. (2010)

where a set of selected 24–30 amino acid peptide fragments e.g., originating from EM13, EmII/3, EmAgB8/1 and EmAgB8/2 were tested with a small panel of sera from AE and CE patients. In this evaluation, however, none of these peptides analyzed, or combinations thereof, exhibited a practically relevant diagnostic sensitivity and specificity. These data give rise to doubts that short antigenic peptide sequences can provide a realistic basis for future developments in order to further improve specific immunodiagnosis of AE and CE cases.

For AE patients, a life-long followup upon surgical and/or and chemotherapeutical intervention is recommended. This is particularly the case because recurrence of the infection has been observed nearly two decades after surgery (Ammann et al., 2004). In contrast to CE, in AE serological assays are available to assess the efficacy of treatment but these followups have to be performed in combination with periodic imaging of the patients (Siles-Lucas and Gottstein, 2001; Ito and Craig, 2003). For example, a decline of *Echinococcus*-specific IgE and IgG4 levels in serum of AE patients turned out to be associated with good prognosis and absence of recurrence upon surgery and chemotherapy (rev. in Gottstein et al., 2014). Furthermore, re-emergence of specific IgG4 antibodies was indicative for recurrence of the infection (rev. in Gottstein et al., 2014). These findings were confirmed at least to a certain extent in an immunoblot assay where serum IgG4 from AE patients with progressive disease exhibited distinctive reactivity with an *E. multilocularis* antigen pattern appearing on the blot as 26, 18, 16, and 12 kDa bands (rev. in Siles-Lucas and Gottstein, 2001). Analogously, regression of AE from an active to a stable and cured stage was found to be associated with a progressive reduction of *E. multilocularis* metacestode-specific IgG1, IgG3, and IgE responses (Huang et al., 2014). At the same time, respective IgG2 and IgG4 reactivity remained comparably high in stable and progressive AE cases, and dropped in the cured form of the disease only (Huang et al., 2014).

While the assays mentioned above undoubtedly need further evaluations regarding their suitability in follow-up examinations of posttreatment AE patients, the Em18-ELISA, and probably to a minor extent also the Em2^{plus} ELISA containing Em18-derivative EmII/3–10, have already gained a certain acceptance at least in those labs that are specialized on diagnosis of human echinococcosis. Extensive evaluations of these assays revealed that serum anti Em18 antibody levels rapidly decline to undetectable concentrations upon complete surgical resolution of the parasite infection (Ammann et al., 2004; Tappe et al., 2010; Bresson-Hadni et al., 2011).

In a very recent study, two *E. multilocularis* HF antigen proteoforms representing 6- and 8-kDa peptides from EmAgB3 were identified by immunoproteome array (Ahn et al., 2015b). Recombinant EmAgB3 was expressed in *E. coli* as glutathione transferase (GST)-tagged protein and the GST tags were removed by using thrombin for site-specific proteomic cleavage. In immunoblot assays, this antigen revealed a high sensitivity (90.9%) for sera from active-stage AE patients and a high specificity (98.5%) for sera from patients with nonparasitic hepatic lesions, other liver invasive helminthiases and blood donor controls. More importantly however, recombinant EmAgB3 turned out to be a promising immunodiagnostic tool for posttreatment monitoring of AE. While the large majority (>90%) of sera from patients with early or advanced disease scored highly positive in the recombinant EmAgB3-based immunoblot none of the AE patients treated with albendazole for >1 year exhibited visible immunoreactivity in this assay. As demonstrated in experimental murine infections, the immune response to this antigen strongly correlated with parasite viability and AE progression. This finding supported the authors' expectation that EmAgB3 may become a relevant biomarker for serological assessment of AE patients. However, although EmAgB3 and other native or recombinant *E. multilocularis* antigens are gaining increasing importance in follow-up serology of AE, respective data still have to be complemented by additional diagnostic parameters in order to achieve a reliable posttreatment monitoring of the disease. Here, particularly Fluorodeoxyglucose (FDG)-positron emission tomography (PET) imaging has to be mentioned as the best complementary method currently available (e.g., rev. in Gottstein et al., 2014).

While serology is well established in diagnosis of human AE, this methodology is not yet routine in diagnostic or epidemiological assessment of *E. multilocularis* infections in animal intermediate hosts. It is general knowledge that rodents are the natural intermediate hosts of *E. multilocularis* but most of the corresponding studies relied on ultrasound, and/or) immunohistological and molecular methods (polymerase chain reaction, PCR) for detection of the parasite. This was exemplified in epidemiological surveys that demonstrated the prevalence of the parasite within mouse populations from highly endemic regions of Switzerland (Gottstein et al., 1996; Schmitt et al., 1997). Basically the same technologies have been applied in small cohort studies or investigations of individual cases that allowed direct demonstration of metacestodes in liver tissue from various animal intermediate hosts such as beavers (Janovsky et al., 2002), monkeys (Rehmann et al.,

2003; Bacciarini et al., 2004, 2005; Rehmann et al., 2005), hares (Chaignat et al., 2015), chinchillas (Staebler et al., 2007), squirrels (Staebler et al., 2007), and dogs the latter known to act as both definite and aberrant intermediate hosts (Deplazes and Eckert, 2001; Conraths and Deplazes, 2015). In the last few years however, serology turned out to be suited to replace, or at least complement, imaging procedures as well as immunohistology and PCR in diagnosis of AE at least in some of the animal species listed above.

As far as immunodiagnosis of AE in beavers is concerned, availability of a conjugate specific for beaver immunoglobulin to detect antigen–antibody complexes in serological assays represented a major issue for a long time. This problem could be solved by producing via immunization of hens a batch of egg yolk IgY antibodies against partially purified beaver IgG and subsequently covalently coupling alkaline phosphatase to these antiisotype antibodies (Gottstein et al., 2014b). In a first attempt, the diagnostic operating characteristics of this conjugate was tested by Em18-and Em2-ELISA using panels of sera from Swiss and Austria beavers representing either confirmed (by histology and/or PCR) AE cases or animals negative for AE. Here, Em18-and Em2-ELISAs conventionally used for diagnosis of human AE yielded irrelevant (0% for Em18) to moderate (46% for Em2) sensitivities indicating the insignificance of these two assays in serology of AE in beavers. Conversely, immunoblot assays including electrophoretically separated EmVF as antigen and using the same conjugate for detection of antigen–antibody reaction demonstrated a good diagnostic performance by serological testing of 13 positive and 27 negative beavers. Immunoblot profiles from positive beaver sera contained a predominant immunoreactive 21 kDa band and two minor bands of 19 and 40 kDa. Interestingly, the same bands also reacted with a positive control serum originating from a human AE patient. Based on the detection of an immunoreaction with the 21 kDa band, AE in beavers was diagnosed at a sensitivity of 85% and a maximal specificity of 100%. Here, the strong and specific immunoreactivity of the AE-positive sera with a 21 kDa band facilitated unambiguous interpretation of the test results. As recently confirmed in a similar investigation, this excellent practicability makes the immunoblot a convenient tool for diagnosis of AE in beavers particularly in complementation to histology, PCR and/or diverse imaging technologies such as laparoscopy and abdominal ultrasound (Campbell-Palmer et al., 2015).

In 2003, gorillas were identified for the first time as intermediate hosts of *E. multilocularis*. Both cases occurred in zoological gardens, namely in Basle (Switzerland) and Wuppertal (Germany), they were associated with massive

formation of lesions in the liver, and had a lethal outcome (Rehmann et al., 2003). Clinical signs as well as macroscopical and microscopical findings after necropsy indicated that the characteristics of the disease in these animals resembled to those described for AE in humans. This case study gave the first opportunity to demonstrate that serology is basically suited to diagnose AE in primates. In fact, testing of sera in an Em2-ELISA provided positive results for both animals. Interestingly, this analysis was successful although antihuman alkaline-phosphatase conjugate instead of primate-specific secondary antibodies was used for detection of the immunoreaction. The Em2-ELISA was also successfully applied for the identification of *E. multilocularis* infections in cynomolgus monkeys (Bacciarini et al., 2004; Rehmann et al., 2005). In these studies, the assay was adapted for analysis of cynomolgus sera in that a commercial anti cynomolgus monkey IgG conjugated to alkaline phosphatase was introduced as secondary antibody in the test system. Despite these promising findings however, routine application of the Em2-ELISA for immunodiagnosis of AE in monkeys still necessitates a careful evaluation of the assay regarding both its diagnostic sensitivity and specificity.

It is known since many years that dogs can become an aberrant intermediate host of *E. multilocularis* (Deplazes and Eckert, 2001; Conraths and Deplazes, 2015). Here, parasitic eggs ingested on contaminated grass or via coprophagy are considered the major source of infection but also auto-infection as consequence of an intestinal infection with adult tapeworms has been proposed (Haller et al., 1998; Staebler et al., 2006). Canine AE is mostly symptomatic, and infection has often a lethal outcome if not efficaciously treated (Skelding et al., 2014; Corsini et al., 2015). In those studies on canine AE published so far, case identification occurred based on positive results in imaging, cytology, histopathology (Oscos-Snowball et al., 2015) and/or PCR (Skelding et al., 2014; Corsini et al., 2015). In a former study, a relatively large number ($n = 30$) of confirmed canine AE cases was serologically tested using ELISAs including a set of seven different antigens (Staebler et al., 2006). This set consisted of *E. multilocularis* antigens EmII/3—10, Em2G11, protoscolex (EmP), excretory/secretory (EmAdE/S) antigen and adult integument (EmAd/I), and *E. granulosus* antigens EgHF and EgAgB. Diagnosis also included detection of circulating antigen Em2G11. In this complex test setup, Em2G11-and EmII/3-10-based assays detected 53 and 50% of AE cases. If specifically applied for diagnosis of animals with large parasite masses and ascites, combined circulating Em2G11 antigen and corresponding antibody detection provided a sensitivity of 77%. Highest

sensitivities, however, were obtained in ELISAs with EmAd/I (97%) and the EmP (93%) antigens and this test combination demonstrated relatively high specificity (100 and 98.7%, respectively) within the control group (n = 76). Conversely, EmAdE/S-antigen, EgHF and EgAgB exhibited relatively low sensitivities (47, 43 and 50%, respectively) and specificities (<84%) by testing the AE-positive and control serum panels outlined above. Interestingly, follow-up serology applicable in four dogs revealed a correlation between the development of the parasite mass and the antibody reactivity patterns in the Em2G11-, EmP- and EmAd/I -ELISAs.

The data presented by Staebler et al. (2006) suggested a combinatorial application of several antigens to achieve an accurate diagnosis of AE in dogs. However, this assumption is challenged by a very recent study that relied on the exclusive use of the Em2-ELISA for a serological investigation of canine AE cases following medical treatment alone or surgery and medical treatment. Here, all confirmed and probable AE cases investigated (n = 10) unambiguously scored positive thus indicating a high sensitivity of the test in canine AE serology. Although this finding was rather promising, further studies will be necessary in order to find out if the Em2-ELISA alone is sufficient for both a reliable immunodiagnosis and a posttreatment serological monitoring of AE in dogs.

3.1.3 In patients with polycystic echinococcosis

As mentioned, very few cases of human polycystic echinococcosis (PE) have been reported in the literature. The two species causing PE, *Echinococcus vogeli* and *Echinococcus oligarthrus*, are much less studied in its antigenic composition than *Echinococcus granulosus* and *Echinococcus multilocularis*. For this reason, in the majority of PE cases the antigen used for its serodiagnosis has been the HF from *E. granulosus* (rev. in D'Alessandro and Rausch, 2008), with the exception of a purified fraction of *E. vogeli* named Ev2 antigen (Gottstein et al., 1995), that could differentiate antibody responses between *E. vogeli* and *E. granulosus* patients but not between *E. vogeli* and *E. multilocularis* patients, although the latter two species are not sympatric and differentiation of the two of them could be done by epidemiological means. Unfortunately, the test using the Ev2 antigen could not be further evaluated and its value as antigenic source should be tested with a higher number of samples to define its usefulness for the specific detection of antibodies in PE patients.

Due to the limitations of HF for the serological diagnosis of PE patients, similar to those found for CE patients, attempts to define the reactivity of

antibodies in PE patients against defined HF antigens (AgB) have been done (de la Rue et al., 2010). Unfortunately, AgB subunits are shared by the different species of the genus *Echinococcus* (Haag et al., 2006), resulting in cross-reactivity of sera from PE, AE and CE patients against the 8 kDa antigen from *E. granulosus*, with a sensitivity of 66.7% against this subunit for PE patients (de la Rue et al., 2010).

3.2 Detection of antigens, lymphoproliferation and detection of cytokines

3.2.1 *In cystic echinococcosis*

An alternative for the diagnosis of CE is the detection of antigens in body fluids. In this sense, antigen detection could be of advantage compared with the detection of antibodies in early stages of infection and for the followup of treated patients, since the decrease of circulating antigens should occur before the decrease of antibodies in cured patients. Efforts to detect circulating antigens in CE patients have been reviewed by Craig and Nelson (1984), Gottstein (1992), Lightowlers and Gottstein (1995), Siles-Lucas and Gottstein (2001) and Zhang et al. (2012).

However, the detection of circulating antigens in CE patients has been hampered by its presence below detection limits in a percentage of patients due to a low release of antigens from the cyst or to the binding of the released antigens to antibodies forming circulating immune complexes (CIC) (D'Amelio et al., 1989). An early example of the low sensitivity of the detection of antigens in CE patients is given by Gottstein (1984). A double-antibody-sandwich-ELISA was developed by immunizing rabbits with *E. granulosus* antigens and further purifying the IgG fraction by affinity immunochromatography and immunosorption with bovine and human sera. From 21 CE patients, only 7 showed detectable antigen levels in serum. In contrast, a hyperimmune serum raised in rabbit against human HF and used to sensitize cells for a coagglutination assay resulted in the positivity of 16 out of 17 sera from surgically confirmed CE patients (Shariff and Parija, 1993). Later, the same group published the use of the same antibodies to sensitize latex particles and use them in a latex agglutination tests for the detection of circulating antigens in CE patients, obtaining 68% sensitivity with patients surgically confirmed or diagnosed by ultrasonography (Devi Chandrakesan and Parija, 2003). Sadjjadi et al. (2009) using a rabbit hyperimmune serum against HF purified in a protein A column could detect circulating antigens by ELISA in 25.7% of surgically confirmed CE patients, with a specificity of 98%. These contradictory results showing a high

variability in the sensitivity of circulating antigen detection are probably attributable to the same reasons found for variable specificity in the detection of antibodies (e.g., clinical variables) or to the presence of higher amounts of free circulating antigens (not in CIC) in some patients compared with others.

Antibodies raised against defined antigens have also been used for the detection of circulating antigens in CE patients. The purified 27.5 kDa protoscolex antigen has been used to raise polyclonal antibodies further used for the detection of antigens in ELISA (Bauomi et al., 2015). Nevertheless, sensitivity of the assay was still below acceptable standards (52.5%), and with a specificity of 75%. Additionally, a number of monoclonal antibodies against several antigens of *E. granulosus* have been developed, but only some of them have been tested for the detection of antigens in CE patients (rev. in Siles-Lucas and Gottstein, 2001). A low sensitivity (50%) was found combining the reactivity of four monoclonal antibodies against Ag5 and AgB for the detection of circulating antigens in serum (Liu et al., 1993).

Of importance is the development of tools for the detection of antigens in body fluids other than serum and easier to collect. Urine and saliva samples have been used, the first after antigen concentration by different means, mainly ammonium persulfate precipitation. The coagglutination tests developed by Shariff and Parija (1993) was used for the detection of antigens in urine of CE patients, showing that 43.7% of surgically confirmed patients and 60% of patients diagnosed by US had detectable antigens in urine (Ravinder et al., 2000). Sunita et al. (2011) approached the detection of antigens in urine and saliva from CE patients by ELISA with an anti HF rabbit hyperimmune serum, showing 52 and 24% sensitivity for urine and saliva samples, respectively, and a specificity of around 80%. These authors pointed out that antigen positivity in urine was significantly higher ($p < .05$) in patients with hepatic cysts than that in extrahepatic cysts. Similar sensitivity rates (around 50%) were found by Swarna and Parija (2012) with a hyperimmune serum raised against a total homogenate of a single human cyst, used for the detection of antigens in urine by dot-ELISA and immunoblot. Derived from this work, a sandwich ELISA was developed using rabbit polyclonal antibodies against a 24 kDa antigen specifically recognized in urine samples, resulting in 70% sensitivity in the detection of antigens in urine from surgically confirmed and US diagnosed CE patients (Chaya and Parija, 2013).

Several methods to release and concentrate the antigens complexed with antibodies have been used to enhance the sensitivity of antigen detection

tests in CE patients, and applied for their followup. Acidic treatment (0.2M glycine/HCl) of patients serum results in the best release of complexed antigens, since all the sera of 30 confirmed cases of CE had detectable levels of antigens in acid treated sera (Craig, 1993). Treatment of serum with 3% polyethylene glycol (PEG) and detection of antigens with peroxidase conjugated hyperimmune human hydatid IgG (Fab) was used to successfully differentiate current from previous hydatid infection and to detect antibody false negative/low responders in Great Britain and Turkana CE patients (Craig, 1986). The same approach was used to demonstrate the decline of circulating antigens in a number of CE patients after 1–4 months of surgical cyst removal (Craig, 1986) and later used to monitor a reduced number of CE patients (n = 6) under drug treatment, showing that circulating antigens could provide additional measures of the persistence of parasitic activity (Awar et al., 1991). In the same line, Ravinder et al. (1997) showed the die-off of circulating antigens in surgical and drug treated patients in a coagglutination test similar to that used by Shariff and Parija (1993), although the number of patients under study was limited. Consistent with these observations, Ferragut et al. (1998) used a hyperimmune serum raised in rabbits against HF and further purified against HF, to demonstrate the absence of detectable circulating antigens in CE patients cured after surgery. Importantly, these authors found that some patients with relapses showed a rise in detectable antigens in serum as early as two months after surgery, thus circulating antigens being a marker of early cyst development (Ferragut et al., 1998). Similarly, increased circulating antigens were of prognostic value in some severe CE cases where levels remained high and/or increased (Bonifacino et al., 2000). Detection of specific antigens with monospecific antibodies has been also done in acidic treated serum, resulting in high sensitivity (Kanwar et al., 1994).

Combination of the detection of antibodies and circulating antigens has been shown to increase the sensitivity of each test alone (e.g., Barbieri et al., 1994). This is of importance for false-negative patients in antibody tests that could be positive in an assay for the detection of antigens, although this should be further demonstrated with an extended number of samples.

Carbohydrate antigens have been also investigated in body fluids from CE patients. A molecule related with the antigen 19-9, a tumour marker, was found to be present in a percentage of CE patients [13 out of 19 (68.4%); Pfister et al., 2001], and elevated in AE patients, compared to CE patients, which could be significant for the differential diagnosis of the two diseases.

Few examples can be found in the literature regarding circulating antigen detection in infected animals for the diagnosis of CE. Antibodies raised against the 27.5 kDa protoscoleces antigen applied to detect antigens in CE patients were also tested in serum from naturally infected sheep by ELISA, showing similar sensitivity than for patients (60%) and a specificity of 88% (Bauomi et al., 2015). Urine from *E. granulosus* experimentally infected sheep was also tested for the detection of antigens by using an anti HF hyperimmune serum raised in rabbit (Ghorbanpoor et al., 2006). The use of this hyperimmune serum in the coagglutination test described by Ravinder et al. (2000) and in the counter-immunoelectrophoresis tests described by Shariff and Parija (1993) resulted in the detection of antigens in 100% of infected sheep at 3 months post infection, showing better sensitivity than the detection of antibodies in haemagglutination (Ghorbanpoor et al., 2006). Antigens and antibodies were detected along the infection course from the first month post infection until the end of the experiment (4 months post infection) in a nonsymmetric Gaussian distribution with the peak at 3 months post infection, showing that antibodies in serum and antigens in urine of infected animals decrease after 3 months post infection. Whether this test could be useful for the demonstration of CE in chronic infections in sheep is still a matter of concern.

In summary, circulating antigen detection could be of use for the diagnosis of antibody-negative patients, but diagnostic samples should be treated before testing them to release the antigens forming complexes with antibodies and making them available for its detection. Thus, antigen detection could be more time-consuming than antibody detection and a common method for antigen release should be produced. On the other hand, there are enough evidences to postulate the antigen detection as a good method for the followup of treated patients that show detectable antigen levels at the beginning of their treatment, especially for surgical treatment. Nevertheless, further studies should be done to check the potential influence on the different antigen levels in body fluids, ideally urine, of several factors that have not been assessed to date like parasite genotype, and number, location, size and stage of cysts. A second matter of concern is the specificity of the sera raised to detect the antigens by the different authors, which should be further investigated. The application of specific recombinant antigens for the obtainment of specific and sensitive antibodies could be of great help to improve future tests based in the detection of circulating antigens.

The detection of antigens has been also approached in cystic material obtained after aspiration of cyst contents. Additional to the demonstration

of solid material (protoscoleces) by microscopy when present, the fluid can be subjected to reaction with a number of antibodies specifically developed for this purpose. Coagglutination with an anti HF hyperimmune serum (Shariff and Parija, 1993) has been also used for the detection of specific antigens in fluid from human cysts with 100% sensitivity by Parija et al. (1996). The same antiserum was applied in a latex agglutination test with a sensitivity of 100% in material obtained from six human fertile cysts (Devi Chandrakesan and Parija, 2003).

Demonstration of *E. granulosus* antigens in the cystic fluid to establish the aetiological agent has been also done with more specific reagents. Polyclonal antibodies obtained against the purified AgB and labelled with peroxidase were used to test cyst fluid samples from surgically confirmed CE patients in a dot-ELISA which can be performed in 10 min, showing 100% sensitivity and specificity, although the number of tested samples was limited, especially those used to test the specificity of the assay (Wang et al., 2002). Antibodies against a 24 kDa hydatid antigen detected in urine of CE patients was also tested for the detection of antigens in aspirated fluid by sandwich-ELISA, showing 100% sensitivity (Chaya and Parija, 2013). A monoclonal antibody against AgB (A11B1) has been used in a sandwich-ELISA to detect specific antigens in HF obtained from patients' cysts (Ortona et al., 1995), although the authors do not specify which subunits of AgB are reactive against this antibody. A second monoclonal antibody against Ag5 has been also used in ELISA for the detection of the corresponding antigen in liver cyst material obtained after fine needle aspiration biopsy (Paul and Stefaniak, 1997). In this assay, cysts containing protoscoleces were 100% positive, but only a small percentage of cyst material from nonfertile cysts was positive, highlighting the need for the definition of potentially expressed antigens depending on cyst fertility to develop sensitive tests for both nonfertile and fertile echinococcocal cysts. This is also applicable for the detection of antigens in different cystic stages, since expressed antigens are qualitatively and quantitatively different along cyst development (Ahn et al., 2015a). Thus, the detection of specific antigens in aspirated cyst material could be an alternative to microscopy, although an extended number of samples from different *E. granulosus* cyst types and from cystic lesions of different aetiologies should be tested ideally with polyclonal antibodies with high sensitivity and specificity.

The majority of the developed antibodies have been also used for the detection of antigens in fluid collected from animal cysts with variable but promising results.

Cellular immune responses have been extensively studied in CE patients. Specific proliferation of peripheral blood mononuclear cells (PBMCs) from CE patients in response to in vitro stimulation with HF or semipurified fractions of this antigen has been shown by several authors (e.g., Siracusano et al., 1988; Ausiello e al., 1989; Shweiki et al., 1992; rev. in Gottstein, 1992; Kharebov et al., 1997). Importantly, some patients with low antibody titres demonstrated high proliferative responses, thus PBMC proliferation assay could be an adjunct to serology in the diagnosis of CE patients. Specific fractions of the HF containing either Ag5 or AgB seem to be the most indicated to trigger CE patients' PBMC specific proliferation in vitro (Profumo et al., 1994; Ioppolo et al., 1996).

The use of PBMC proliferation for the followup of treated patients seems to be unsuitable, since proliferative responses remain high in cured patients over a long period after successful removal of the hydatid cyst or after drug treatment, although at lower levels than in patients before treatment, immunosurveillance by specific antibodies may be of more practical use than antigen-specific proliferation of cells in monitoring treated patients (Ioppolo et al., 1996; Bonifacino et al., 2000; rev. in El-On, 2003).

PBMC have been also used for the detection of cytokines produced after in vitro specific stimulation, generally with HF, of cells from CE patients in comparison with healthy controls. Riganò et al. (1995a, 1996) showed that PBMC from CE patients produced higher amounts of IL-4 and IL-5 than controls after specific antigen stimulation. Higher production of IL-10 and IFNg was also detected in CE patients compared with controls, but differences were not statistically significant. IL-6 production did not show differences between patients and controls. High IL-4, IL-5 and IL-10 production correlated with high levels of specific IgE and IgG4 in CE patients. The high production of IL-5 and IL-10 of PBMC from CE patients after antigen stimulation, both with HF from *E. granulosus* and *E. multilocularis*, was confirmed at mRNA level by Fauser and Kern (1997). Similarly, high production of IL-4 and its correlation with high IgE and IgG4 antibody levels were found in PBMC from CE patients after stimulation with the AgB (Riganò et al., 2001).

Levels of cytokines have been also investigated as an adjunctive to serology in seronegative CE patients. Intriguingly, these patients did not produce IL-5 and produced scarce IL-4 and IL-10 cytokines after antigen stimulation of PBMC, compared with seropositive patients, suggesting an inadequate Th2 cell activation due to unknown factors in seronegative

patients that could result in the lack of production and thus absence of detectable levels of antibodies in this group of patients (Riganò et al., 1998).

A relationship between the level of defined cytokines and some clinical variables has been observed by several authors, although the number of studied patients was low and detected differences should be further investigated. A relationship between an increased production of IFNg and the presence of inactive cystic lesions, compared with the levels produced by patients with active or transitional cysts, has been shown (Riganò et al., 2004). Similarly, patients with partially calcified lesions showed decreased levels of both circulating IL-8 and IL-12 in serum (Amri et al., 2008). These authors also showed a potential relationship between lower levels of those two cytokines in serum in patients with single cysts or extrahepatic cysts, compared with patients with multiple cysts and cysts in liver. Later, lower levels of cytokines in patients with single extrahepatic cysts compared with hepatic or multiple cysts was extended to other chemokines, including IL-5, IL-12, IL-16 and IFNg (Mezioug and Touil-Boukoffa, 2009). An attempt to find a relationship between IL-4, IL-10, IL-12 and tissue necrosis factor-alpha (TNFa) cytokine levels in serum and cyst activity in CE patients was published by Tamarozzi et al. (2010). Only IL-4 showed to be significantly higher in patients with CE3b cysts, both in its median levels and in the total number of positive patients, and the authors stated that the analysis of serum cytokine levels for the above-mentioned chemokines is not useful as marker for cyst activity. In a subsequent publication from the same working group (Piccoli et al., 2012), the levels of IFNg, IL-4, IL-13 and IL-10 were measured ex vivo in serum samples of CE patients compared to healthy controls. Authors point out that serum levels of cytokines in CE patients are variable regardless cyst stage, and that the ELISA technique for the detection of circulating cytokines is limited by its low sensitivity and specificity. A limitation of this and similar studies is the small sample size which is insufficient to evaluate intergroup differences.

More recently, additional insights in the specific production of cytokines in CE patients depending on the cyst stage have been published with an extended number of patients. Zhang et al. (2015) tested 64 patients for the levels of IL-4 and IL-21 in serum by using a cytometric bead array. They found that the concentrations of IL-21 and IL-4 in the serum were significantly increased in CE1, CE2, and CE3 groups, compared with the group of patients with inactive cysts. Interestingly, there was also a correlation between the different IgG subisotypes and the cyst activity, showing that IgG1 and IgG4 are increased in patients with active and transitional cysts

and IgG2 and IgG3 were elevated in patients with inactive cysts. Similarly, stimulation of whole blood cells or of PBMC from 46 CE patients with AgB and measurement of the levels of produced IL-4 by ELISA showed that IL-4 levels were statistically higher in patients with active cysts than with inactive cysts (Petrone et al., 2015a). The same working group showed by flow-cytometry that IL-2^+TNF-α^+Th2^+ triple-positive and TNF-α^+Th2^+ double-positive specific T-cells associate with cyst biological activity, after stimulation with AgB of CD4^+ T-cells isolated from CE patients (Petrone et al., 2015b).

Regarding cytokine patterns in followup patients, in a study performed with drug treated CE patients, responders to the drug treatment produced significantly less IL-4 and more IFNg than nonresponders, in parallel with a decrease in IgE and IgG antibodies against AgB, while IL-5 and IL-6 levels did not show an association with the outcome of the drug treatment (Riganò et al., 1995b, 1996). Later, elevated IL-12 p40 mRNA levels in PBMC after antigen stimulation were related with successful drug treatment in CE patients, who also showed an increase in IFNg and TNFa mRNA levels, while high levels of IL-4 correlated with therapy failure (Riganò et al., 1999).

Also for the definition of changes in the cytokine patterns in follow-up patients, Hernández-Pomi et al. (1997) compared the cytokine production by PBMC after antigen stimulation in vitro from CE patients with primary infection and relapses. These authors, similar to Riganò et al. (1995a,b) showed that IL-2, IL-5 and IFNg levels were higher in patients than in controls and correlated with high IgE and IgG4 levels in serum. Although a tendency to an increased production of IL-5 and decreased production of IFNg was detected in patients with relapses compared with patients with primary infections, differences were not statistically significant (Hernández-Pomi et al., 1997). The dynamics of cytokine changes has been also studied in CE patients in followup after surgical treatment. Serum levels of TNFa, IL-1b, IL-2R, IL-6 and IL-8 were determined by chemiluminescent ELISA in CE patients before and after treatment, and compared with those in healthy donors (Refik et al., 2005). Only IL-6 was found to be elevated in all CE patients before treatment, and a positive correlation between this cytokine and the levels of C-reactive protein in serum was found in those patients, observation that was extended to IL-17A by Mezioug and Touil-Boukoffa (2012). Levels of IL-6 decreased in cured patients, compared with a single patient who showed a relapse, in which IL-6 levels were still high (Refik et al., 2005). Levels of other

Th2 cytokines have been also shown to decrease in cured patients after surgery, including IFNg and IL-4 (Mezioug and Touil-Boukoffa, 2009). The relationship between serum cytokine levels measured by ELISA and the outcome of drug treatment was also evaluated by Naik et al. (2016) in 50 CE patients. From these, 82% showed detectable levels of IL-4, 74% of IL-10 and 50% of IFNg before treatment. Those responding to treatment showed significantly decreased levels of IL-4 and IL-10 after 2 years of treatment.

In summary, lymphoproliferation assays are of very limited use in CE, although cytokine detection could be of applicability, especially in noncomplex assays like serum cytokine detection in ELISA. The detection of cytokines in serum is thus a potential tool for the diagnosis and clinical management of CE patients. Nevertheless, none of the different cytokine markers investigated until now can be still considered applicable for routine diagnosis. Variable sensitivity have been found for different cytokines, and although high IL-4 levels are the most frequent finding in CE patients, this cytokine is absent in some of them. Some of the described cytokines could be also of use for the followup of CE patients. Nevertheless, cytokine detection is not of use as an adjunctive to serology, since seronegative patients are also negative in cytokine tests. In this setting, cytokine detection seems to perform similar to antibody detection and if some advantages should be mentioned for cytokines detection *vs*. antibody detection, these would be probably more related with the decline of specific cytokine levels in a shorter time than the decline of antibody levels in follow-up patients after cure and the definition of cyst activity, although this should still be further investigated.

3.2.2 In alveolar echinococcosis

Detection of circulating *E. multilocularis* antigens was considered as realistic concept to directly demonstrate presence of the parasite in AE patients with active lesions before and after treatment (Gottstein et al., 2014). For followups, these circulating markers could e.g., be applied in combination with other circulating markers such as cytokines and chemokines. At least to our knowledge, however, current literature does not provide noteworthy evidence about the applicability of this concept in diagnosis of AE.

In contrast, antibodies specific for *E. multilocularis* have successfully been used for directly demonstrating parasite material in various tissue samples from AE patients. In this respect, MAbs against the serologically highly

relevant antigen Em2 have to be mentioned in first place as tools for a specific immunohistochemical diagnosis of AE (Deplazes and Gottstein, 1991; Wang et al., 2010; Barth et al., 2012). Surprisingly, however, in this respect only one of these MAbs, namely MAb Em2G11, was adequately evaluated regarding its potential to specifically detect *E. multilocularis* material by immunohistochemical means (Deplazes and Gottstein, 1991; Barth et al., 2012). Here, species-specific binding of MAb Em2G11 was confirmed in that the antibody was revealed to bind to metacestode tissue from various *E. multilocularis* isolates but not to any other helminth isolates (e.g., *E. granulosus*, *E. vogeli*, and others) tested (Deplazes and Gottstein, 1991). Apart from some applications in basic research in AE, MAb Em2G11 was initially produced in order to provide a tool for affinity-purification of Em2 antigen for conventional AE serology (Em2-ELISA, see above). However, MAb Em2G11 also served as a reagent for the development of both a direct immunofluorescence assay and a sandwich-ELISA for specifically tracing Em2 antigen in cytological samples from AE patients (Deplazes and Gottstein, 1991). As described in the following paragraphs, this MAb represents an excellent tool for specific detection of *E. multilocularis* metacestode material in both immunohistology and immunocytology.

As reported by Barth et al. (2012), MAb Em2G11-based immunohistology on metacestodes grown in Mongolian jirds resulted in a strong staining of both the laminated and the germinal layers, the calcareous corpuscles as well as of the fluid inside the cysts. Conversely, protoscoleces did not exhibit reactivity with MAb Em2G11 but immunostaining of a dense layer surrounding the protoscoleces was observed.

In the same study, the excellent operating characteristics of MAb Em2G11 in immunohistology was assessed by analysis of 96 archived paraffin-embedded tissue sections from patients with suspected AE or CE, respectively. Using classical histological criteria based on staining of sections with haematoxylin and eosin (H&E) and periodic acid-Schiff reaction (PAS) 49 (51%) of the samples were classified as AE, and 47 (49%) as CE. From these 96 samples, 12 were considered difficult to diagnose because of atypical immunostaining features in tissue samples from bone lesions or tissue samples with necrotic lesions, or strong fragmentation of the LLs without context to the surrounding. Among those exhibiting strong fragmentation, three samples were aspirates from a liver lesion, a muscle lesion and from the bile duct. In this relatively large-scale analysis, metacestodes in sections from all suspected AE cases were strongly positive. Here, unambiguous diagnosis was even possible in those histological specimens that were characterized by a

loose distribution of very small fragments from the LL. In addition, the use of MAb Em2G11 in immunocytology allowed confirmatory diagnosis of *E. multilocularis* in an aspiration fluid from a liver lesion of an AE patient. In contrast, no immunostaining at all of specimens from suspected CE cases was detected. Furthermore, the importance of immunocytology as confirmatory diagnostic assay was demonstrated in a case where metacestode material was detected in a fine-needle aspiration from the multicystic mass in the pancreas (Diebold-Berger et al., 1997). This study also showed that cytology may be a highly valuable method for diagnosis of AE especially in cases were the risk of misdiagnosis exists as consequence of rarely occurring extrahepatic locations of *E. multilocularis* metacestodes.

The hepatic development of *E. multilocularis* metacestodes in an AE patient is essentially determined by the host's local immune response involving granuloma formation around the hepatic lesions (Gottstein et al., 2014). Depending on the characteristics of the response, immunological reactions lead to resistance as being recognizable by the presence of calcified lesions representing 'aborted' metacestodes, or result in either slowly growing metacestode tissue in 'normal' AE patients exhibiting symptoms 5—15 years post infection, or massive metacestode proliferation often associated with an immunodeficient status of the patient. Due to the obvious existence of a close correlation between distinct immune reactivity patterns and the manifestation of the disease, immunological parameters obtained from lymphocytes proliferation or cytokine asssays are considered as potential markers for posttreatment monitoring and prognostic diagnosis of AE in human patients. Mainly for practical reason, this diagnostic concept was essentially focussed from the beginning on testing of blood and/or plasma samples that can be collected from patients by minimal-invasive procedures.

In a preliminary view of identifying potential markers for AE, the production of cytokines and chemokines in PBMC of AE patients versus controls following in vitro stimulation with different *E. multilocularis* metacestode antigen fractions was assessed in various studies. In this respect, a very early study from the mid 90s was aimed at the analysis of the specific proliferation of the PBMC from 36 patients with AE, and 23 controls, stimulated by a crude preparation of *E. multilocularis* antigen, Em2 antigen or a protoscolex-derived antigen (Nicot et al., 1994). Here, the authors most importantly found that irrespective of the antigen used for stimulation, the PBMC exhibited a significant proliferation even in those AE cases that were serologically negative. As subsequently assessed by analysis of

mRNA levels in PBMC, *E. multilocularis* infections seemed to activate a Th2-immune response leading to increased levels of IL-3, IL-4, IL-10 and most strikingly of IL-5 (Sturm et al., 1995). However, since two patients who experienced radical surgery and a patient exhibiting a stable course of the disease during chemotherapy, did not show this cytokine pattern a crucial role particularly of IL-5 in the manifestation of human AE was suggested. This assumption was confirmed by findings from Jenne et al. (1997) revealing that the expression of IL-5 and other Th2-type interleukin mRNAs occured at a significantly higher frequency in patients with progressive AE as compared to other patients and negative controls.

In an analogous investigation, PBMC from AE patients exhibited a comparatively depressed release of proinflammatory cytokine IL-12 and Th2-type chemokine CCL17 and a suppression of tumour necrosis factor (TNF)a (Hübner et al., 2006). Interestingly, production of IFNg was increased when PBMC from AE patients were compared to controls under the same cultivation conditions. This effect was associated with a higher release of the Th2-type chemokine CCL22 (macrophage-derived chemokine, MDC) thus suggesting that *E. multilocularis* infections also generate proinflammatory immune responses. In a similar approach, Vuitton (2003) could demonstrate that especially patients with abortive AE exhibited a strong lymphocyte proliferative response following in vitro stimulation of PBMC with *E. multilocularis* antigens. In the same study, the author additionally found a correlation between increased number of $CD4^+$ T cells within the granuloma and an abortive, or at least slow, development of the metacestodes. In abortive cases however, PBMCs were characterized by a reduced production of IL-10 (Godot et al., 2000). Conversely, patients with progressive AE specifically exhibited a spontaneous secretion of IL-10 by the PBMC (Godot et al., 2000). A fluorescence activating cell sorting analysis of PBMC from AE patients and healthy controls upon in vitro stimulation with crude *E. multilocularis* antigen detected an enhanced expression of IL-10 in $CD8^+$ T cells from the patients (Kilwinski et al., 1999). In AE patients, $CD8^+$ T cells are generally present in hepatic granulomas (Manfras et al., 2004) and more importantly this cell type seems to be predominant in inefficient granulomas in severe AE cases (Vuitton, 2003). Since the cells producing IL-10 at the highest level were found to be in close contact with the metacestode structures an involvement of the parasite itself in inhibition of the cellular immune effector mechanism probably leading to increased parasite growth was suggested (Haraga et al., 2003).

Further efforts in finding immunological parameters relevant for human AE were made by the investigation of the inflammation-associated regulatory functions that may influence the outcome of the disease. For example, AE was found to be accompanied by a significant suppression of the cellular release of proinflammatory IL-31 and IL-33 (Huang et al., 2014). Furthermore, Tuxun et al. (2015a,b) provided preliminary evidence that increased local and peripheral expression of proinflammatory IL-23 (as well as IL-9, Toll-like receptor 2, transcription factors PU.1 and IRF-4) might be involved in both the modulation of the tissue-infiltrative growth of *E. multilocularis* metacestodes and the persistence of the parasite in the human host). In another study focussed on cytokine patterns associated with AE, PBMC-derived CC and CXC chemokine profiles of patients with progressive, stable, or cured AE versus health controls were compared upon their stimulation with *E. multilocularis* antigens in vitro (Kocherscheidt et al., 2008). Here, all groups of AE patients consistently exhibited elevated levels of inflammation-associated chemokines MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCLMIP-5 whereas levels from other chemokines were clearly diminished. Furthermore, Lechner et al. (2012) studied the proinflammatory IL-17 cytokine family members and their common receptors in AE patients. By demonstrating divergent cellular production profiles and plasma concentrations of individual IL-17 family members, Th-17-type IL-17A levels were similar in all groups of AE patients. Interestingly, IL-17B production was increased and IL-17F production was depressed in all AE patient groups as compared to healthy controls. IL-17-secreting Th17 cells (Pang et al., 2014; Ma et al., 2014), besides other immunological effectors such as regulatory T-cells (Treg) (Pang et al., 2014), were also supposed to facilitate long-term survival of the parasite by taking into account their putative regulatory function in Th1/Th2 cell balance, immune tolerance and hepatic tissue inflammation. Conversely, modulation of both regulatory and inflammatory Th1 and Th2 cytokines and chemokines by *E. multilocularis* antigen may also lead to a mixed profile that might limit parasite growth and reduce periparasitic tissue and organ damage in the host (Hübner et al., 2006). In a further study, progression of the disease was shown to coincide with increased levels of regulatory IL-27, antiinflammatory CXC chemokine SDF-1, and CC chemokines eotaxin-1, 2, and 3 involved in eosinophil granulocyte attraction (Huang et al., 2014). In addition, the regulatory T-cell effector fibrinogen-like protein-2 (FGL-2) has been identified as a candidate marker for discrimination between AE patients with active and

inactive lesions. Here, preliminary data indicated that the soluble form of FGL-2 might be increased in sera from patients with AE as compared to control sera from healthy blood donors but its prognostic potential in monitoring of the disease still remains to be elucidated (Gottstein et al., 2014).

In conclusion, the different studies outlined above provide preliminary information about those immunoregulatory processes that determine the status of AE in the human host. Respective data may indicate that Th2 responses, and more specifically IL-5 expression as well as IL-10 secretion by PBMC are associated with a progressive course of AE in humans. Accordingly it is feasible, that quantification of IL-5 and IL-10 in blood or plasma (or serum) samples may have a certain potential to complement Em18-based serology as diagnostic tools for the identification of highly active AE in humans. Based on other findings outlined above, a similar diagnostic potential may be attributed to some proinflammatory cytokines and chemokines that turned out to be differentially regulated in relation to different stages of AE. Unfortunately however, none of the investigations addressing these processes could be performed with statistically significant numbers of patients in order to achieve a solid evaluation of the individual immunological parameters regarding their true diagnostic value as markers for the different manifestations of the disease. Apart from that, the diagnostic application of these parameters would be complicated by the extreme complexity of the anti AE immune response involving pro- and antiinflammatory immune reactions exhibiting mixed Th1/Th2-type cytokine and chemokine patterns (see above). Due to this complex immunological situation associated with AE, lymphocyte proliferation assays with PBMC following stimulation with *E. multilocularis* antigen as well as analyses of distinct cytokine and chemokine patterns in stimulated PBMC, or in blood (including corresponding plasma and serum) samples without previous stimulation, still have to be considered impractical procedures for posttreatment monitoring and prognostic diagnosis of human AE cases.

4. IMMUNOLOGICAL DIAGNOSIS OF THE DEFINITIVE HOSTS

In the following, an overview on the immunological diagnosis of definitive hosts is presented. Information on the use and performance of microscopical methods used for the detection of the parasite in definitive hosts can be found in chapter "Echinococcosis: Control and Prevention" by Craig et al. (2016).

Antigens of *Echinococcus* spp. may interact with the immune system of the definitive host and thus cause the production of specific antibodies (Jenkins and Rickard, 1985; Craig et al., 2003). The adult parasites penetrate deeply between the villi into the Lieberkühn crypts, where they attach with the rostellar hooks and suckers to the epithelium (Thompson et al., 1995). Despite this close contact between the parasite and the host and potential lesions, which might be caused by the rostellar hooks, there pathological changes to the mucosa can hardly be observed. Only minor changes such as a slight infiltration, local flattening of epithelial cells and increased mucus production have been reported. The fact, that there is only a mild, if any, local reaction to the infection of the definitive host with juvenile intestinal and adult stages of *Echinococcus* spp. may explain the lack of sensitivity of the serological tests that were developed for the detection of specific antibodies in these animals. Another problem is the persistence of antibodies after elimination of the parasites, which limits the diagnostic specificity of the serological tests (Gottstein et al., 1991; Gasser et al., 1993; Craig et al., 2003).

When antibodies against the Em2 antigen were tested to assess the suitability of the antigen for diagnostic purposes in foxes, the main definitive host of *E. multilocularis*, a sensitivity of 12–60% was found and animals without intestinal infection with *E. multilocularis* were also positive in the test (Gottstein et al., 1991). It has been proposed that the detection of circulating anti Em2 antibodies by ELISA may be useful for primary screening of fox populations for exposure to *E. multilocularis*, but it is important to note that seroprevalence estimates obtained with this approach do not correlate with prevalence estimates obtained by methods that aim at directly investigating the intestinal infection with *E. multilocularis* (Deplazes and Eckert, 1996).

Excretory/secretory molecules released from the scolex region of *E. granulosus* may induce an antibody response in dogs (Jenkins and Rickard, 1986 a,b). Serum antibodies (IgG, IgA and IgE) against an *E. granulosus* protoscolex antigen preparation were detected in experimentally infected dogs using an ELISA within 2–3 weeks post infection (Gasser et al., 1988, 1990, 1992, 1993). A basic component of 27 kDa and an acidic component of 94 kDa were defined in both excretory/secretory and somatic protoscolex antigens and were specifically identified by 95 and 62% of 21 sera from *E. granulosus*-infected dogs (Gasser et al., 1989).

A sensitivity of 73% (16 out of 22 dogs) was found with the ELISA with protoscolex or oncosphere antigen among naturally infected dogs in south-east Australia, but a statistically significant correlation with the worm burden was not observed (Gasser et al., 1988). When the *E. granulosus* protoscolex antigen preparation was used in Uruguay (Gasser et al., 1994) and Kenya (Jenkins et al., 1990), the sensitivity ranged between 35% and 40% relative to worm identification at necropsy or after arecoline purgation (Craig et al., 1995). The specificity of the test was higher (70 − 95% or even ≥97%).

Furthermore, for example in dogs, such tests were not able to differentiate between intestinal *E. multilocularis* infections and alveolar echinococcosis in dogs (Staebler et al., 2006).

In conclusion, serological screening using crude parasite antigens or affinity-purified Em2 antigen has been considered unsuitable for a reliable diagnosis of intestinal *E. multilocularis* infections because of the poor correlation between the presence of antibodies in the serum and worms in the intestine. Further investigations may utilize existing or develop new recombinant antigens and assess their potential for a reliable diagnosis of *Echinococcus* spp. infections in definitive hosts (Carmena et al., 2006; Zhang and McManus, 2006). Currently, diagnosis at necropsy or after arecoline purging (by the sedimentation and counting technique or the intestinal scraping technique and their variants) or coproantigen- and coproPCR-based methods represent better alternatives (Zhang and Mc Manus, 2006; Conraths and Deplazes, 2015; Craig et al., 2015).

Antigen detection in faecal samples (coproantigen) of definitive hosts may have the potential to replace necropsy techniques and arecoline purging. At the same time, such techniques could have the advantage over serology in definitive hosts for detecting current infections (Craig et al., 2015). To identify suitable sources of coproantigens, somatic extracts or excretory/secretory antigens of adults or protoscolices were screened and polyclonal or monoclonal antibodies produced against promising candidates (Alan et al., 1992; Benito and Carmena, 2005; Morel et al., 2013).

Several coproantigen tests have been established for the detection *E. granulosus* (mainly in dogs) and *E. multilocularis* (mainly in foxes, but also in dogs and cats (Table 2).

Coproantigen tests originally developed for the diagnosis of *E. granulosus* showed cross-reactivity with *E. multilocularis* (Allan et al., 1992;

Table 2 Available coproantigen enzyme linked immunosorbent assay tests for the detection of *Echinococcus* sp. in DH

Test	Target parasite[a]	Sens (%)	Spec (%)	Cross react[a]	Reference
R anti EgW	*Echinococcus granulosus* s.l.,	83	96	Th	Allan et al. (1992), Craig et al. (1995), Buishi et al. (2005)
R anti EgWES	*E. granulosus* s.l.	87	98	Th	Deplazes et al. (1992)
Mab EgWES, EmA9	*E. granulosus* s.l.	100	96	Th, Tm	Malgor et al. (1997), Nonaka et al. (2011)
R anti EgPxES	*E. granulosus* s.l.	78.4	93.3	?	Benito and Carmena (2005)
Mab EgES, EgC1/EgC3	*E. granulosus* s.l.	100	100	Th	Casaravilla et al. (2005)
R anti EgWWES, S anti EgWFT	*E. granulosus* s.l.	92	80	*Taenia*	Huang et al. (2008)
R anti EgW	*E. granulosus* s.l.	92	86.5	*Taenia*	Pierangeli et al. (2010)
Mab Eg9ES	*E. granulosus* s.l.	86.5	86.4	*Taenia*	Morel et al. (2013)
R anti EgW[b]	*E. granulosus* s.l.	60	93	*Taenia*	Huang et al. (2014), commercial kit[b]
R anti EmPaES R anti EmW C anti c-AG-IgG-c	*Echinococcus multilocularis*	84[c]	94	*Taenia*	Deplazes et al., 1999
R anti EmWES	*E. multilocularis*	89	93	*Taenia*	Yimam et al. (2002)
R anti EmWES	*E. multilocularis*	87	70	*Taenia*	Sakai et al. (1998)
n.d.[d]	*E. multilocularis*	72	96	*Taenia*	Reiterová et al. (2005)
R anti EgW	*E. multilocularis*	55	70.6	Th	Allan et al. (1992)

C, chicken antibodies; *c-AG-IgG-c*, coproantigen-IgG complexes; *Mab*, monoclonal antibodies; *PaES*, preadult excretory/secretory; *Px*, protoscolex; *R*, rabbit antibodies; *S*, sheep antibodies; *Th*, Taenia hydatigena; *Tm*, T. multiceps; *W*, adult somatic; *WES*, adult excretory/secretory; *WFT*, adult freeze-thaw; ?, cross-reactivity unknown.

[a]Most tests are genus-specific, i.e., they do not reliably differentiate between *E. multilocularis* and *E. granulosus* s.l.

[b]From Xinjiang Tiankang Animal Husbandry Biotech Co., Ltd., Urumqi, China.

[c]40% in faecal samples of animals with worm burdens ranging from 4 to 20 to 100% in samples with worm burdens of 520–60,000 parasites.

[d]n.d. = no data; commercial test Chekit Echinotest (Dr. Bommeli AG, Liebefeld-Bern, Switzerland).

Data partially extracted from Craig, P., Mastin, A., van Kesteren, F., Boufana, B., 2015. *Echinococcus granulosus*: Epidemiology and state-of-the-art of diagnostics in animals. Vet. Parasitol. 213, 132–148; modified

Deplazes et al., 1992). When polyclonal chicken and rabbit or mouse monoclonal antibodies produced against *E. multilocularis* E/S or integument antigens were used in the ELISA, the sensitivity could be improved, but the test remained *Echinococcus*-genus specific. At present, no test is available that utilizes highly genus-specific monoclonal antibodies or polyclonal antibodies directed to defined antigen fractions. As a consequence, the tests remained difficult to reproduce on a large scale and over time. There is a commercialized ELISA kit, which includes a rapid test for the detection of *E. multilocularis* coproantigens (EKITTO, In-Vio Science Inc., Tokyo, Japan), but this test may not be specific in areas with high prevalences of *Taenia* spp. Furthermore, three *Echinococcus*-specific coproantigen tests have been commercialized in China (Huang et al., 2013), but an evaluation for *E. multilocularis* infections has so far not been made available.

Canine echinococcosis due to *Echinococcus granulosus* can be detected with reasonable sensitivity and good genus specificity ranging from 85 to $\geq$ 95% in coproantigen ELISAs (78−100%; Allan et al., 1992; Benito and Carmena, 2005; Buishi et al., 2005; Craig et al., 2015). Detection of prepatent infection is possible, but with limited sensitivity (Deplazes et al., 1991; Jenkins et al., 2000). If cross-reactions occur, they appear to be frequently caused by *T. hydatigena*, a common taeniid of dogs (Malgor et al., 1997; Morel et al., 2013). The sensitivity of *E. granulosus* coproantigen ELISAs is associated with the worm burden of the parasite (Malgor et al., 1997; Fraser et al., 2002; Buishi et al., 2005). *E. granulosus*-infections of low intensity may thus lead to false-negative results in coproantigen-ELISAs (Allan and Craig, 2006).

The use of *E. multilocularis* coproantigen tests has recently been reviewed (Conraths and Deplazes, 2015). *E. multilocularis* coproantigens appear to be highly resistant to degradation in the environment (Stieger et al., 2002) and some seem to be heat resistant (Nonaka et al., 1996). Similar chemical properties have been described for *E. granulosus* coproantigens (Craig et al., 2015). Characterization of a major *E. multilocularis* coproantigen isolated by the monoclonal MAbA9 (Sakai et al., 1998) led to the discovery of an integumental glycoprotein with unique O-glycosylation expressed in experimentally activated protoscoleces and in adult worms of intestinal origin (Hülsmeier et al., 2010).

E. multilocularis coproantigens can be detected during prepatency and patency in dogs, foxes, raccoon dogs and cats. They disappear within a few days after the elimination of *E. multilocularis* from the host

(Sakai et al., 1998; Deplazes et al., 1992, 1999; Kapel et al., 2006; Al-Sabi et al., 2007).

The sensitivity of coproantigen detection was 83.6% in 55 foxes with worm burdens of 4—60,000 as determined by the Sedimentation and Counting Technique (SCT) in an area highly endemic for *E. multilocularis*, but reached 93.3% in 45 foxes with more than 20 worms. It therefore seems that this test identified those animals that harboured approximately 99.6% of the total number of adult *E. multilocularis* in the tested fox population (Deplazes et al., 1999). If the fact that SCT misses around 20% of infected animals, mainly those with low worm infections may allow to estimate that the sensitivity of the coproantigen ELISA can reach approximately 60% and is strongly dependent on the distribution of the worm burden in the fox populations. The sensitivity of the same coproantigen ELISA for patent *E. multilocularis* infections, as validated by PCR using 17 environmental fox samples, was 88% (Stieger et al., 2002). A recently performed *meta*-analysis on four studies comparing a coproantigen ELISA with (a modified) SCT (Reiterova et al., 2005; Deplazes et al., 1999; Sakai et al., 1998; Yimam et al., 2002) revealed a sensitivity of 82% (95% CI 74—88%) and the specificity of 89% (95% CI 75—96%) of the results of the studies are combined (Casulli et al., 2015).

5. MOLECULAR (DNA-BASED) DIAGNOSIS

The required properties of DNA-based tests for *Echinococcus* diagnosis should measure the actual infection status with high sensitivity and specificity, possibly be able to detect infections at *intra vitam* and postmortem, be suitable for mass-screening, enable DNA quantification, are safe for laboratory personnel and are cost-effective. With the advent of molecular and biochemical approaches for the detection of parasites, different methods were developed during the last 30 years in order to identify *Echinococcus* variants (strains) from animal and human hosts. Such studies, mainly based on PCR approaches, were used for the identification of species, genotypes and haplotypes (hereby used to describe the genetic microvariants) observed within *E. granulosus sensu stricto* (s.s.) and for the differential diagnosis of *E. granulosus sensu lato* (s.l.) and *E. multilocularis*. PCR-based methodologies have found a broad applicability for detection, population studies and epidemiological investigations of this genus, mainly because their analytical sensitivity permits the analysis of nuclear and

mitochondrial gene regions from fresh, frozen, ethanol fixed and paraffin-embedded parasitic material. These techniques were also used on different analytes such as eggs, worms, protoscolices or germinal layer from metacestodes, and from heterogeneous matrices such as soil, vegetables, host intestinal mucosa and faeces. In this section we review the main genetic markers used for the detection of *E. granulosus* s.l., *E. multilocularis* and the tools used for the analysis and identification of variation between/within these species.

5.1 Type of markers

Key loci used for the identification of *Echinococcus* spp. are located within the mitochondrial genes or nuclear ribosomal DNA (rDNA). Mitochondrial DNA (mtDNA) has been widely used for the identification of closely related species because it is a multicopy genome, and is thus more useful in detecting DNA that may be fragmented or present in low quantities particularly in complex matrices (such as faeces and paraffin embedded formalin fixed samples). Due of its relatively rapid rate of evolution mtDNA has been used to differentiate between genotypes/species belonging to *Echinococcus* spp. Furthermore, as mtDNA is haploid, allele haplotypes can be determined unambiguously simplifying sequencing and analysis. An additional advantage is that mtDNA is maternally inherited and does not recombine. Nuclear rDNA has also been used as a source for PCR markers for species and genotype identification representing a wide multigene family of hundreds of tandemly repeated sequences within specific chromosomes.

Mitochondrial DNA is composed by 12 protein-coding genes: adenosine triphosphatase subunit 6 (*atp*6), cytochrome c oxidase complex (*cox*1-*cox*3 subunits), cytochrome b (*cob*) and nicotinamide dehydrogenase (*nad*1–*nad*6 and *nad*4L subunits). Among others, the most used targets for *Echinococcus* are: *cox*1, *nad*1, *cob* and rRNA genes. Nuclear rDNA markers used for the identification and characterization of *Echinococcus* genus include the internal transcribed spacer (ITS), external transcribed spacer (ETS) and the 28S rRNA gene. In particular, two genes have been targeted for the detection of *E. multilocularis* and *E. granulosus* worms in fox/dog intestines and faeces: the U1 snRNA gene and the mitochondrial 12S rRNA.

Microsatellite markers for studying the population genetics and transmission biology of *Echinococcus* have been developed as single (U1snRNA, EgmSca 1, EgmSca 2, and EgmSga 1) or multiloci (EmsB)

(Rosenzvit et al., 2001; Bartholomei-Santos et al., 2003; Nakao et al., 2003; Bart et al., 2006). A summary of markers and molecular methods used for the detection of *Echinococcus* specimens in intermediate, definitive and human hosts can be found in the Supplementary Table 1.

5.2 Type of material and polymerase chain reaction assays used

The diagnosis of *Echinococcus* in faecal samples retrieved from dogs or foxes as well as from other definitive hosts is dependent on the stage of infection (prepatent or patent), and may be hampered by host diet and intermittent shedding of eggs in faeces. PCR-based methodologies performed on DNA extracted directly from relatively abundant parasitic material such as worms, eggs and metacestodes usually result in the successful amplification of diagnostic products regardless of the chosen assay and markers. On the other hand, working with complex matrices such as faeces for example poses serious problems on the marker used and the PCR methodology due to the presence of inhibitors and scanty parasitic material. In fact, DNA amplification can be performed by nested PCR or real-time PCR in order to maximize the sensitivity of the assay when faecal samples are tested. Moreover, DNA amplification can be performed by conventional-PCR targeting a single sequence or, in a multiplex-PCR, in which multiple DNA sequences can be amplified and detected (Monnier et al., 1996; Dinkel et al., 1998; Trachsel et al., 2007; Boubaker et al., 2013). However, the use of multiplexes for the detection of *Echinococcus* spp. DNA in faeces is usually characterized by reduced sensitivity.

5.3 Sensitivity of polymerase chain reaction assays

Due to the large variability in both the DNA extraction methods and DNA amplification techniques, it is difficult to compare and assess diagnostic sensitivity of the various assays described for the detection of *Echinococcus* spp. infection in the definitive host. Consequently, limited data is available regarding the sensitivity of the molecular-based tests as demonstrated in a systematic review by Casulli et al. (2015). In fact, a lack of standardization of diagnostic methods detecting *Echinococcus* specimens may also cause variation in sensitivity and specificity between laboratories. To increase the sensitivity, larger volumes of faeces are required, but this is often hampered by the DNA extraction method. In addition, inhibition of the PCR may result in false negative results, which in turn will affect prevalence rates. A solution to this problem is extracting DNA from sieved taeniid eggs

or using an internal control (Mathis et al., 1996). It is important to note that coproPCR approaches may however exhibit decreased sensitivity and are therefore unable to detect prepatent infections. Conversely, this problem is overcome through the use of DNA-fishing. In this sense, Isaksson et al. (2014) evaluated the sensitivity of Magnetic-Capture (MC) PCR using the sedimentation and counting technique (SCT) as a reference standard. In that study sensitivity was evaluated as 88% compared to an SCT positive panel, and 95.7% considering samples with more than 100 worms (Isaksson et al., 2014). PCR provides no information regarding worm burden, and quantitative PCR gives information only on the relative amount of DNA in the analyzed sample. Difficulties in quantifying DNA in a given faecal sample relate to worm lysis, the presence of immature worms that do not release eggs and mature worms that are shedding eggs discontinuously. Similar to SCT, PCR is an expensive and laborious technique however the automation of processes in recent years such as those now available for DNA extraction as well as the reduction in the costs of reagents will enormously simplify the approach of this methodology.

5.4 Sample preparation, DNA extraction and amplification

At least three steps are included in the diagnostic procedure for the detection of this parasite: sample preparation, DNA extraction and specific amplification of *Echinococcus* DNA followed by visualization and measurement of the PCR products. Various methods exist for the different steps. Precautions must be strictly followed when using samples originating from definitive hosts (intestines, faeces, eggs, worms) for personnel safety. Samples have to be frozen at −80°C for 7 days in order to achieve thorough deep-freezing in order to inactivate the eggs (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2016; http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/).

Echinococcus specimens can be collected from the environment (tissue or eggs contained in faeces or dispersed in the soil), metacestode tissue from animal or human hosts (hydatid cysts) and canid definitive hosts (eggs, worms and parasitic tissue in intestines). These samples can be preserved in ethanol (>70% v/v), frozen at least at −20°C or in paraffin-embedded tissues. In this latter case, depending on the formalin-fixation step, the amplification of long fragments of DNA (usually more than 500bp) is not always feasible because of protein cross-linkage.

Several laboratory techniques can be used to isolate and concentrate eggs, worms and parasitic material from animal definitive hosts, such as sequential

sieving and flotation, sedimentation and counting technique (SCT), segmental sedimentation and counting technique (SSCT), intestinal scraping technique (ITS) and shaking in a vessel technique (SVT).

Regarding human hosts, depending on the clinical management of CE and AE, several options are available to isolate metacestode material. AE infection in humans is characterized by the absence of protoscoleces and fluid-filled liquid cysts and cyst layers are usually collected by Fine Needle Biopsy (FNB) during diagnosis or therapeutic interventions such as open surgery or liver transplantation. FNB is also an important tool for the differential diagnosis of AE/CE in the hepato-gastroenterology, but is discouraged due to the potential spillage of HF potentially inducing severe anaphylactic reactions and secondary CE within the abdomen. In CE, the membranes in toto are usually available through surgical techniques, such as laparotomy and laparoscopy. These tissues are also available from percutaneous interventions by aspiration such as puncture-aspiration-injection-reaspiration (PAIR) or *catheter* drainage such as catheterization technique (CaT), modified catheterization technique (MoCaT), percutaneous evacuation (PEVAC) and percutaneous abscess drainage (PAD).

Various DNA extraction procedures exist depending on the matrix (intestines, faeces, soil, vegetables) and parasitic analyte (eggs, cysts, worms) investigated. For DNA extraction, the principal method consists of the classic phenol-chloroform DNA extraction with alkaline lyses step and organosolvent extraction (for procedures see pioneering work of Bretagne et al., 1993; Monnier et al., 1996); as well as the use of commercial DNA isolation kits (Al-Sabi et al., 2007; Ni et al., 2014); and DNA fishing/magnetic capture method (for procedures see Isaksson et al., 2014; Øines et al., 2014).

Echinococcus DNA can be obtained from faecal matrices by three different procedures. The first is the concentration of taenid eggs by a combination of sequential sieving and flotation (Mathis et al., 1996). This approach only retrieves particles of a size close to that of taenid eggs. However, detached segments of worms such as proglottids will not be detected. On the other hand the method can handle large sample sizes (3–20 g). After concentration, the eggs are digested by alkaline lysis and DNA is extracted. Often the extraction is done by using a Boom-silica spin column kit. Taenid eggs concentration, firstly developed for *E. multilocularis* (Mathis et al., 1996) was also used on *E. granulosus* (Cabrera et al., 2002; Stefanic et al., 2004) or both species (Trachsel et al., 2007).

DNA extraction can be also performed directly from faeces (Dinkel et al., 1998; Knapp et al., 2014). This method generally cannot handle more than a maximum of 0.5 g, but will extract all taeniid DNA and also DNA from other organisms present in the sample. This approach, firstly developed for *E. multilocularis* (Bretagne et al., 1993; Monnier et al., 1996; Dinkel et al., 1998) was also used on *E. granulosus* (Abbasi et al., 2003) or both *E. granulosus* and *E. multilocularis* (Boufana et al., 2013).

DNA fishing method/magnetic capture can be used for the selective extraction of taeniid DNA by the means of a more or less specific hybridization probe connected to magnetic beads, Magnetic Capture (MC) (Isaksson et al., 2014; Øines et al., 2014). This method developed for *E. multilocularis* surveillance, is also able to identify the parasite during the prepatent period. The probe will hybridize to the taeniid DNA target selectively, thus excluding the huge amounts of bacterial DNA present in faeces. This method can handle 3 g of sample material, but could be automatized and optimized for the use of up to 10 g of faeces. A higher amount of faecal sample can be used during the concentration of taenid eggs or the DNA fishing method/magnetic capture, with less risk of inhibition of the PCR, thus potentially increasing the sensitivity of the test. These methods selectively enrich the target, allowing more material to be used in the assay. The advantage of enrichment is that a large part of the PCR inhibitory substances are effectively removed. A comprehensive comparison between these two approaches was reported by Øines et al., 2014.

When DNA extraction is to be performed using worms, single worms can be handled under a stereo microscope, washed three times with distilled water and lysed in 10 μl of 0.02N NaOH at 95°C for 10 min. The lysate can then be directly used as template for PCR.

Regarding DNA extraction from the environment, soil samples can be passed through a 4 mm^2 mesh to remove course debris and suspended in KOH, afterwards eggs can be concentrated by flotation in $NaNO_3$ solution (density of 1.35 g/cm^3) (Shaikenov et al., 2004) or can be washed in 0.05% Tween 80 and concentrated by sequential sieving and flotation in $ZnCl_2$ solution (density of 1.4 g/cm^3) (Szostakowska et al., 2014). Fruit, vegetable and mushroom samples can be washed in 0.05% Tween 80 and eggs concentrated by sequential sieving and flotation in $ZnCl_2$ solution (density of 1.4 g/cm^3) (Lass et al., 2015).

DNA can be easily extracted from ethanol (>75% v/v) preserved metacestode tissue retrieved from human and animal hosts. Protoscoleces are the

tissue of choice when performing such DNA extractions. Germinal layers can be also used for these purposes. Parasitic material (protoscoleces and germinal layers) should be washed three times in phosphate-buffered saline at pH 7.2, centrifuged for 3 min at 3000 × g, discharging the liquid phase and the tissues are then used for DNA extraction.

Echinoccocus spp. parasitic material may also be fixed in formalin solution (usually 10% formalin solution may contain 3.7% formaldehyde as well as 1−1.5% methanol). The resulting chemical reaction leads to cross-links between nucleic acids, between proteins, and between nucleic acids and proteins. Sections of 10 μm in thickness can be cut from formalin-fixed, paraffin-embedded tissue (FFPTs) blocks using a microtome. Sections can be deparaffinized using xylene (10 min at 37°C) with subsequent rehydration steps in 100%, 90%, 80% and 70% ethanol, according to the protocol of Schneider et al. (2008). Commercial kits can be used for DNA extraction from deparaffinized blocks, eventually applying a longer step with Proteinase-K (Schneider et al., 2008; Ito et al., 2010; Simsek et al., 2011). More recently, commercially dedicated kits for direct DNA extraction from FFPTs were developed. Due to the degradation of DNA, short fragments, no longer than 200/300 bp, were successfully amplified using mitochondrial genes such as 12S rRNA, *cox*1 and *nad*1 (Schneider et al., 2008; Ito et al., 2010; Simsek et al., 2011). Following sample collection and DNA extraction, a number of different molecular methods can be used for DNA amplification. Box 2 provides some suggested key molecular approaches to detect *Echinococcus* spp.

Box 2 Suggested Molecular Key-Approaches to Detect *Echinococcus* specimens

- Conventional PCR and sequencing of the mitochondrial gene *cox*1 (460 bp) for species/genotypes identification of strains belonging to *Echinococcus* (Bowles et al., 1992).
- Conventional PCR and sequencing of the mitochondrial gene *cox*1 (880 bp) for species/genotypes identification and deep studies on genetic diversity of strains belonging to *Echinococcus granulosus* sensu stricto (Nakao et al., 2000).
- Multiplex PCR for a quick identification of the majority of species/genotypes belonging to *Echinococcus* (Boubaker et al., 2013).
- Multiplex PCR for the differentiation of eggs belonging to *Echinococcus granulosus*, *Echinococcus multilocularis* and *Taenia* spp. (Trachsel et al., 2007).

5.5 Polymerase chain reaction methods and new approaches

PCR is the method of choice for parasite identification, molecular epidemiological studies and confirmatory purposes, although several traditional biochemical and molecular approaches have been used in the past such as PCR-RAPD (Random Amplification of Polymorphic DNA), PCR-RFLP (Restriction fragment length polymorphism) (Bowles and McManus, 1993a; Gasser and Chilton, 1995; Xiao et al., 2006) a Southern Blot hybridization approach (McManus, 1997), PCR-SSCP (Single strand conformation polymorphism) (Zhang et al., 1999) and ddF (Dideoxy fingerprinting), displaying genetic variability in mtDNA fragments within and among populations of *E. granulosus*.

Conventional and robust approaches have been used for genus/species detection such as conventional-PCR, nested-PCR to test faecal samples or multiplex-PCR for a more differential detection of *Echinococcus* spp. Along this path, sensitive approaches involving PCRs and sequencing have been developed to detect variability within species and genotypes (Bowles et al., 1992; Bowles and McManus, 1993b; Nakao et al., 2000). Sequencing is a laborious approach which needs advanced competencies but it is the most sensitive means for detecting genetic variation and species identification of *Echinococcus*.

More recently, affordable and easy to use approaches such as LAMP (loop-mediated isothermal amplification method), were developed and tested (Salant et al., 2012; Ni et al., 2014 Wassermann et al., 2014). LAMP is a perfect tool to use in low resource settings endemic for alveolar and cystic echinococcosis because DNA can be amplified using a simple water bath avoiding the need for complex instruments. However, this is a system that is prone to the introduction of false positives due to its high sensitivity.

Modern approaches involving single-locus or multiloci microsatellite analysis (Rosenzvit et al., 2001; Bartholomei-Santos et al., 2003; Nakao et al., 2003; Bart et al., 2006) have been developed to study the genetic diversity, structure of parasitic population and the geographical origin of *E. granulosus* and *E. multilocularis* variants.

Real-time PCR (qPCR) (Dinkel et al., 2011; Knapp et al., 2014) offers several advantages over conventional PCR for the detection of parasitic infections, including increased sensitivity and specificity, reduction in reaction time and a quantitative estimate of the amount of DNA in the sample. This quantification however may not be related to the real burden of infection

Box 3 Approximate Working Intensity per Person per day to Analyze *Echinococcus* Specimens, According to Conraths and Deplazes (2015)

- Conventional-PCR or Multiplex-PCR with sieving procedure for egg isolation from faeces: 40–80 samples (person/day) depending on taeniid prevalence (for procedures see Mathis et al., 1996; Trachsel et al., 2007);
- Nested-PCR for total DNA isolation from faeces: around 70 samples (person/day) (for procedures see Monnier et al., 1996; Dinkel et al., 1998);
- qPCR for total DNA isolation from faeces: 70 samples (person/day) (for procedures see Dinkel et al., 2011; Knapp et al., 2014);
- MC-PCR with manual or automated DNA fishing from faeces: 70 or 240 samples (person/day), respectively (for procedures see Isaksson et al., 2014).

because wild carnivores excrete variable quantities of faeces, depending on the availability and quality of food.

In addition Real-time PCR with high-resolution melting (qPCR, HRM) has become a sensitive genotyping method, based on the characteristics of thermal denaturation of the amplicons (Rostami et al., 2013; Santos et al., 2013; Safa et al., 2015). This method has a higher performance compared to the classical DNA melting curve analysis. HRM is performed using a fluorescent double-stranded DNA dye that can be used in fully saturating conditions.

New combined approaches like the DNA fishing/magnetic capture followed by qPCR show high sensitivity and high specificity, especially with worm burdens >100 worms. It can be performed using partial automation, making it well-suited for nationwide *E. multilocularis* surveillance programmes (Isaksson et al., 2014; Øines et al., 2014). Supplementary Table 1 shows the various methods used to amplify DNA from *Echinococcus* specimens from definitive, intermediate hosts, and human patients, while in Box 3 the approximate working intensity per person per day required to perform these techniques is reported.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/bs.apar.2016.09.003

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CHAPTER FOUR

The Echinococcoses: Diagnosis, Clinical Management and Burden of Disease

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Abstract

The echinococcoses are chronic, parasitic diseases that are acquired after ingestion of infective taeniid tapeworm eggs from certain species of the genus *Echinococcus*. Cystic echinococcosis (CE) occurs worldwide, whereas, alveolar echinococcosis (AE) is restricted to the northern hemisphere, and neotropical echinococcosis (NE) has only been identified in Central and South America. Clinical manifestations and disease courses vary profoundly for the different species of *Echinococcus*. CE presents as small

to large cysts, and has commonly been referred to as 'hydatid disease', or 'hydatidosis'. A structured stage-specific approach to CE management, based on the World Health Organization (WHO) ultrasound classification of liver cysts, is now recommended. Management options include percutaneous sterilization techniques, surgery, drug treatment, a 'watch-and-wait' approach or combinations thereof. In contrast, clinical manifestations associated with AE resemble those of a 'malignant', silently-progressing liver disease, with local tissue infiltration and metastases. Structured care is important for AE management and includes WHO staging, drug therapy and long-term follow-up for at least a decade. NE presents as polycystic or unicystic disease. Clinical characteristics resemble those of AE, and management needs to be structured accordingly. However, to date, only a few hundreds of cases have been reported in the literature. The echinococcoses are often expensive and complicated to treat, and prospective clinical studies are needed to better inform case management decisions.

1. CYSTIC ECHINOCOCCOSIS

1.1 Introduction

Cystic echinococcosis (CE), which historically has also been known as 'hydatid disease' or 'hydatidosis', is caused by metacestodes of different species of a small tapeworm belonging to the *Echinococcus granulosus* sensu latu complex inhabiting the small intestine predominantly of dogs and other canines. Of these *E. granulosus* sensu strictu is the most widely distributed. These parasites have sylvatic life cycles, often involving wild carnivores and ungulates, and domestic life cycles, usually involving dogs and farm livestock. The latter transmission cycle that is the most common and poses the greatest threat to human health. CE has a worldwide geographical distribution and occurs on all continents, except Antarctica (Jenkins et al., 2005). There may be in excess of one million people currently living with CE at one time (Craig et al., 1996; WHO fact sheet No377). The WHO has included CE in its strategic roadmap for 2020 (Second WHO report on neglected tropical diseases, Geneva, 2013), and efforts are underway to address the burden and impact of CE in selected countries (National Chinese Program on Echinococcosis Prevention and Control, 2010–2015).

Humans are accidental hosts for metacestodes of *E. granulosus* s.l., and are not known to play a role in parasite transmission. A systematic review found that CE prevalence tends to be higher in females and to increase with age (Budke et al., 2013). The incubation period and clinical picture depend on the organ(s) involved. The liver and lungs are primarily affected, but cysts can occur in any organ system. One or more well-delineated spherical cysts can cause symptoms or may be an incidental finding during routine

diagnostic imaging. The presence of cyst(s) is a key diagnostic feature for CE, and imaging techniques are indispensable. For abdominal lesions, ultrasound imaging (US) is the method of choice, but computed tomography (CT), magnetic resonance imaging (MRI) or radiography may be indicated depending upon the features and location of the cyst(s). The WHO—Informal Working Group on Echinococcosis (WHO-IWGE) classifies hepatic cysts based on pathognomonic US features, where cysts are defined 'active' (CE1 and CE2), 'transitional' (CE3, including CE3a and CE3b) and 'inactive' (CE4 and CE5) (WHO-IWGE, 2003). An additional stage, cystic lesion (CL), was included to identify undifferentiated cysts found in community-based studies or during mass screening. Notably, CL cysts are not included as a type of CE and require additional evaluation. Another diagnostic method is serology (as discussed in Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017). While serology remains unreliable as a primary diagnostic tool, it can play a confirmative role when CE is suspected (Brunetti et al., 2010).

Historically, surgery was the primary treatment for CE. However, recent advancements allow for a stage-based therapeutic approach. Depending on the site of infection and cyst stage, four approaches, or combinations thereof, are recommended: (1) medical therapy (benzimidazoles); (2) minimally invasive percutaneous sterilizing techniques; (3) surgery and (4) watch-and-wait (Junghanns et al., 2008; Brunetti et al., 2010). Before the 1980s puncture of hepatic echinococcal cysts was considered contraindicated due to the perceived risks of anaphylaxis and secondary dissemination of metacestodes. However, such serious complications did not develop in some patients whose cysts were unintentionally punctured (Mueller et al., 1985; Akhan et al., 1998a, 2002) or who received percutaneous treatment as a new minimally invasive technique (Filice et al., 1990). Further evaluation found that dissemination could be minimized by adjunctive treatment with benzimidazoles (BMZ). To date, percutaneous treatments of abdominal CE cysts have been applied safely in thousands of cases worldwide (Neumayr et al., 2011), and this minimal invasive approach is now more common than surgery in some countries (Akhan, personal communication).

Unfortunately, comparative data from studies evaluating different treatment modalities, in particular the cyst stage-based triage of patients, are still lacking (Brunetti et al., 2010). In devising a treatment plan the clinical condition of the patient needs to be carefully taken into account, as well as the technical conditions of the health-care facility, the safety and

effectiveness of the approach and the costs of each method. Whichever treatment is selected, long-term follow-up is mandatory, and patients should be cared by an interdisciplinary team specializing in CE. This not only contributes to significantly better care for CE patients, but also facilitates data collection, with the goal of improving treatment protocols. One initiative that was developed to assist with data collection is the European Register of Cystic Echinococcosis (Tamarozzi et al., 2015).

1.2 Clinical diagnosis and definitions

1.2.1 Metacestodes in the human host

1.2.1.1 Growth, structure and size

After eggs are being ingested by an intermediate host, embryos (oncospheres) hatch, penetrate the gut wall, enter blood or lymphatic vessels and are trapped in internal organs, where they develop into the larval stage (metacestode) of *E. granulosus*. Within the affected organ a single-chambered vesicle is formed, which expands slowly by concentric enlargement. The newly formed cyst contains secretions from both the parasite, including antigenic substances, and the host.

As depicted in Fig. 1A the cyst's morphological structure consists of (1) a host-derived fibrous, i.e., adventitial layer (often previously termed the 'pericyst'); (2) the parasite-derived inner layers composed of a 'thick' outer acellular 'laminated layer (LL)' and a 'thin' inner syncytial 'germinal layer (GL)' and (3) a liquid content or 'hydatid' cyst fluid, which may or may not contain protoscoleces (see Chapter : Biology and Systematics of

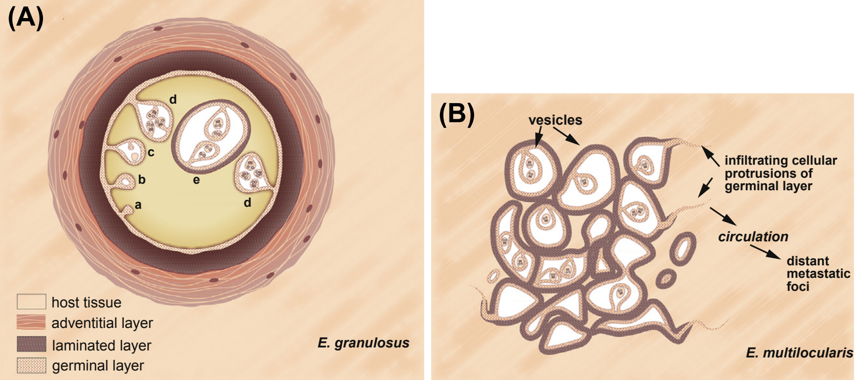


Figure 1 Diagram illustrating the structural differences between the metacestodes of *Echinococcus granulosus* (A) stages in development of protoscoleces and brood capsule, and daughter cysts, and (B) *Echinococcus multilocularis. Courtesy by A. Thompson.*

Echinococcus by Thompson, 2017). Protoscoleces are formed from brood capsules, which budd from the GL. The brood capsules may detach into the hydatid fluid within the cyst or within daughter cysts located inside the main cyst. In some instances brood capsules and/or protoscoleces are not present. Cysts with these properties are regarded as viable but are not infective to a definite host (Rogan et al., 2006). For cysts that contain protoscoleces, each protoscolex may develop into an adult worm if ingested by a definitive host. Within an intermediate host, cysts may also disseminate to other tissues in the event of a cyst rupturing.

1.2.1.2 Natural course of metacestode growth

The natural history of *E. granulosus* s.l., cysts in humans is not fully understood, with current knowledge based on US observations made at mass screenings in endemic areas (Romig et al., 1986; Frider et al., 1999; Wang et al., 2006). However, care must be taken since cysts with morphological aspects which suggest viability may actually be not viable and vice versa (Hosch et al., 2008). In an East African study by Romig et al. (1986), within 12–18 months, among 44 cysts, 29 grew, reaching sizes of 10–150 mm, nine were static, three collapsed and three disappeared, including one cyst with daughter cysts. The yearly growth rate of cysts was highly variable (from no change up to 130 mm/year; 29 mm on average). However, cyst growth was faster and greater in younger patients, especially in children and teenagers, and slower in the elderly. There were no significant differences between genders or between primary and recurrent cysts. Long-term follow-up of asymptomatic patients has shown that most of liver cysts have a very slow and limited growth. In more than half of the CE cysts in a South American study by Frider et al. (1999), there were no changes in cyst size during the 10- to 12-year period of observation. For one-third of cysts, growth was slight (<30 mm), and mean cyst growth in all 14 cases with a prolonged follow-up was 7 mm per year. In the Chinese study, among untreated CE patients, one out of six cysts exhibited a spontaneous resolution within 4 years (Wang et al., 2006). Partially or fully calcified cysts are not uncommon (Hosch et al., 2007). Rogan et al. (2015) developed a model to divide cyst stages into four phases: (1) maturing; (2) stable; (3) unstable and (4) degenerative. Maturing cysts are increasing in size and progressively producing brood capsules and protoscoleces (i.e., acquiring 'fertility'). Stable cysts exhibit little increase in size and remain viable whether or not they produce protoscoleces. Unstable cysts are submitted to 'stressing events' (e.g., fissure or rupture of the GL that may manifest by the detachment of membranes at US). This may lead to

degeneration, production of daughter cysts within the main cyst due to the multipotential capability of stem cells located in the GL or dissemination and seeding of other organs/tissues with protoscoleces (Rogan et al., 2015).

1.2.1.3 Cyst localization

Most patients (up to 80%) have a single organ involved and harbour a solitary cyst, while the other 20% of cases involve multiple organ systems (Grove et al., 1976). In primary CE, metacestodes develop from oncospheres which have successfully established in the affected organ/tissue. In contrast, in secondary CE the larval tissue spreads from the primary site to other parts of the body. Secondary CE often occurs after spontaneous or trauma-induced rupture of a cyst or after release of viable parasite material (protoscoleces and/or parasitic stem cells) during invasive treatment procedures. Primary or secondary cysts do not differ in appearance.

The role of parasite species in the location of cysts, if any, is unclear. Conventionally, all infections with CE were attributed to variants of *E. granulosus* (Smyth and Davies, 1974). However, mitochondrial phylogenetic analysis has allowed taxonomists to classify most of the genotypes as new species (Thompson, 2008). For example, *E. granulosus* s.s., *Echinococcus equinus*, *Echinococcus ortleppi*, *Echinococcus canadensis*, *Echinococcus felidis*, some of which have different host preferences, and, apparently, different levels of infectivity and/or pathogenicity for humans (Nakao et al., 2013; see Chapter: Biology and Systematics of *Echinococcus* by Thompson, 2017). Specific tropism of *E. canadensis* to the lung and brain has been suggested (Bardonnet et al., 2002). A systematic review of the literature of human CE indicated that *E. granulosus* s.s. metacestodes preferentially develop in the liver (73.4%) and secondly in the lungs (19.6%). The G6 genotype of *E. canadensis* is reported to affect the liver (54.3%), lungs (25.7%), brain (12.9%) and other organs (7.1%), while the 'European' G7 genotype of *E. canadensis* appears to almost exclusively develop in the liver (98.6%) (Cucher et al., 2016).

1.2.2 Clinical features

After an undefined and variable incubation period, CE may become symptomatic if active cysts exert pressure on adjacent tissue or induce other pathologic events (Ammann and Eckert, 1996). In a considerable number of patients, CE is an incidental finding during imaging examinations made for other reasons. In other situations, CE is diagnosed in asymptomatic patients during community screenings in endemic regions (e.g., Romig et al., 1986; Larrieu et al., 2004; Moro et al., 2005; Del Carpio et al., 2012;

Kilimcioglu et al., 2013). Typically, cysts do not induce clinical symptoms until they have reached a particular size. The diversity of clinical manifestations, associated with a ruptured cyst, is related to the anatomical localization of the cyst, its size and release of antigenic material responsible for systemic hypersensitivity reactions.

For the following anatomical locations, signs and symptoms are described according to the site involved, and relative frequencies (in brackets) are displayed with a reference to a large dataset consisting of up to 16.000 patients (Pawlowski et al., 2001) or to a systematic review of the literature on CE frequency and its associated clinical manifestations (Budke et al., 2013).

Liver: The liver is the most common location for cysts to develop (69–75%). The development of a cyst is slow and usually without specific clinical manifestations. However, mechanical, toxic or septic effects can result in complications (21%). In general, clinical manifestations associated with liver cysts are divers, with patients presenting with abdominal pain, dyspepsia, fever or allergic manifestations, including a rash. Rupture to the biliary tree is a common occurrence (Zargar et al., 1992; Kornaros et al., 1996), presenting with signs of cholangitis and/or bile duct obstruction (Akhan et al., 1994b). Rupture into the peritoneal cavity may result in anaphylactic shock or acute abdomen (Karavias et al., 1996).

Lung: The lungs are the second most common organ affected (17–22%). Multiple cysts occur in approximately 30% of cases, cysts occur bilateral in 20% of cases, and cysts are located in the lower lobes in 60% of pulmonary cases (Ramos et al., 2001). Most cysts are acquired in childhood, remain asymptomatic for a long period of time, and are later diagnosed incidentally on chest radiography (Todorov and Boeva, 2000). Rarely, lung cysts may develop secondary to rupture of a hepatic cyst via the diaphragm. Multiple cysts can also result from haematogenous spreading or from secondary dissemination from a preexisting lung cyst. Intact cysts may cause nonspecific symptoms, such as chest pain, chronic cough and haemoptysis (Santinvanez and Garcia, 2010). Compression of a cyst in the bronchi may result in retention pneumonia, atelectasis or an inflammatory reaction. Patients with ruptured cysts may present with an urticarial rash with or without fever or systemic anaphylaxis. Expectoration of salty material (parasitic membranes), bacterial superinfection and cyst haemorrhage have been reported (Morar and Feldman, 2003).

Spleen: Spleen cysts (1–3%) are accompanied with hepatic or peritoneal cysts in 30% of cases. Splenic cyst growth is insidious, and the cyst can reach giant dimensions or rupture into the peritoneal cavity prior to seeking

treatment. Splenic CE often remains often asymptomatic. However, patients may report discomfort in the left hypochondriac region (Akhan and Koroglu, 2007).

Peritoneum: Peritoneal CE is a rare occurrence and typically results from spontaneous or traumatic rupture of a hepatic cyst in 85% of the cases or is secondary to abdominal surgery (Karavias et al., 1996). Clinical manifestations can be associated with inflammation or with anaphylactic shock and are present as acute abdomen which requires immediate surgical intervention (Vaizey et al., 1994).

Kidneys: Renal cysts (1–4%) normally occur as a primary infection, with cysts located in both kidneys. As renal CE is rather insidious, symptoms are nonspecific (Akhan et al., 1998a). The most frequent clinical manifestations are pain or a 'mass' in the lumbar region. However, haematuria and pyelonephritis with fever have been reported (Zmerli et al., 2001). The rupture of a cyst into the ureter can result in renal colic with a 'hydatiduria' (parasite particles passing with urine).

Bone: Echinococcosis of bones is uncommon (<1%). Cysts can be located in the vertebral spine (50%), long bones, pelvis and rarely in the skull, ribs, sternum or scapula. The vertebral location is the most serious and can result in neurologic complications due to the spinal cord compression. Cysts, in this location, have a case fatality of more than 50% (Zlitini et al., 2001). The most frequent manifestations associated with infection of long bones are pathologic fractures or fistula formation.

Brain and spinal cord: Echinococcal cysts may develop in the brain (<1%). This occurs predominantly in children and young adults (Altinörs et al., 2000; Khaldi et al., 2000). The cysts are usually solitary, sized between 5 and 10 cm in diameter, and are located in the frontal or occipital regions in 65% of cases. Symptoms depend on the location of the cyst and typically develop slowly. A common first sign in children is intracranial hypertension with headache, nausea, vomiting and papilloedema. Young adults may additionally present with seizures, hemiparesis, hemianopsia or speech disturbances (Duishanbai et al., 2010). Spinal CE is associated with a high degree of morbidity and mortality (Akhan et al., 1991; Neumayr et al., 2013a,b).

Heart: Cysts are rarely found in the heart (1%), with the ventricular wall being the most common location (Yalcin et al., 2002; Birincioglu et al., 2013). The patients can present with thoracic pain, dyspnoea and heart palpitations. If a cyst is located in the right side of the heart, serious complications can result from intracardiac rupture of the cyst, resulting in a pulmonary embolism. If the cyst is located in the left side of the heart, neurologic

complications may occur (El Fortia et al., 1998). If the cyst ruptures into the pericardium, pericarditis or cardiac tamponade can occur (Dıaz-Menendez et al., 2012).

Rare sites: Other sites where cysts have been identified include muscle (2%) (Akhan et al., 2007b; Guven et al., 2004), ovaries (<1%), pancreas (0.2%) (Nabi-Yatoo et al., 1999) and the adrenal gland (Akhan et al., 2011). CE has also been described in the thyroid gland, in salivary glands (Akhan et al., 2002) and in the orbit of the eye, resulting in exophthalmia, eyelid ptosis and visual disturbance (Akhan et al., 1998b). Data on cysts of the oromaxillofacial structures have recently been reviewed by Just et al. (2014). A 2013 review also evaluated rare CE cyst locations in Iranian patients (Geramizadeh, 2013).

1.2.3 Diagnostic imaging

Various imaging modalities, including ultrasonography (US), CT, MRI and conventional radiography, are important for the diagnosis of CE (Haliloglu et al., 1997; Polat et al., 2003). These techniques are used for classification, staging, identification of possible complications and monitoring the response to treatment. Metabolic viability assessment may be achieved by analysis of cyst content using high-field ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS).

1.2.3.1 Evolution of the WHO ultrasound classification

Classifications of the various appearances of CE cysts are based on US features obtained from liver scans. When US first became widely available, Gharbi et al. (1981) classified liver cysts into five groups: type I (pure fluid collection), type II (fluid collection with a split wall), type III (fluid collection with septa), type IV (cysts with heterogeneous echogenicity) and type V (cysts with thick walls). In the years that followed, several modifications were proposed (Beggs, 1985; Lewall and McCorkel, 1985; Perdomo et al., 1995; Caremani et al., 1997; Shambesh et al., 1999), with the claims that 'Gharbi's' classification (1) does not adequately reflect the natural history of the disease; (2) is exclusively based on the morphology of the cyst without taking into account cyst viability and (3) does not classify all subtypes of CE. In 1995 the WHO-IWGE evaluated the existing classification schemes for advantages and weaknesses with reference to simplicity, pathophysiological relevance and utility for the follow-up of treated patients. Final agreement was achieved in 2001(Fig. 2), with details of the consented classification were issued in 2003 (WHO-IWGE, 2003).

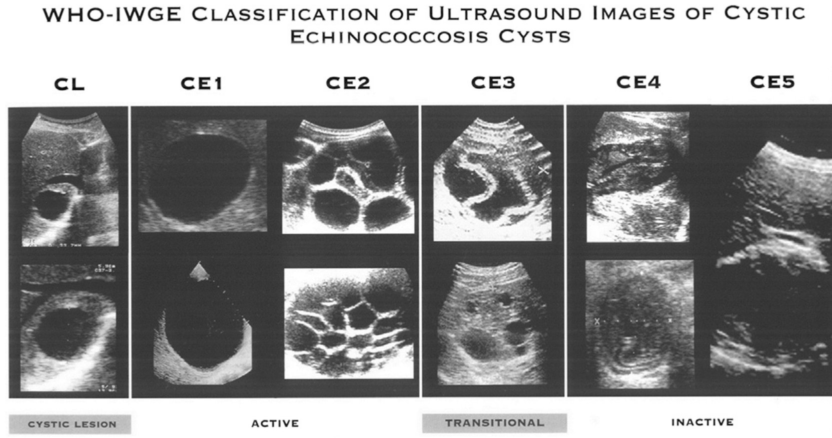


Figure 2 WHO–Informal Working Group on Echinococcosis standardized classification (WHO/CDS/CSR/APH/2001.6).

Two main differences between the WHO-IWGE and 'Gharbi's' classification were (1) the introduction of the category 'cystic lesion' (CL), which accommodates cysts without pathognomonic signs of CE thus necessitating further diagnostic procedures and (2) the reversing of the order of 'Gharbi' type II and III into CE3 and CE2, respectively, to better align cysts considered transitional. Thus the WHO-IWGE classification grouped cysts into three clinical categories: active cysts that are developing and usually fertile (CE1, CE2); transitional cysts that are degenerating but usually still containing viable protoscoleces (CE3) and inactive cysts that have degenerated or are calcified and unlikely to be fertile (CE4 and CE5). For each category, cyst diameter was also considered, with cysts classified as small (<5.0 cm), medium (5 to <10 cm) or large (>10 cm) (WHO-IWGE, 2003).

After publication of the WHO-IWGE classification, discussions continued regarding the natural history of CE cysts and whether the WHO typing and grouping (active–transitional–inactive) were appropriate (Wang et al., 2003; Hosch et al., 2007). In 2008, Junghanns et al. proposed making a distinction between CE3a and CE3b type cysts on the basis of clinical response to percutaneous treatments and/or drug therapy with BMZ (Stojkovic et al., 2009; Nasseri-Moghaddam et al., 2011). High relapse rates were seen in patients with CE3b cysts treated with puncture-aspiration-injection-reaspiration (PAIR). These cysts also had a poor response to treatment with BMZ. The suggested change to the WHO-IWGE classification was officially adopted in 2010 (Brunetti et al., 2010).

Unfortunately, acceptance of the standardized ultrasound classification is rather poor. In 71.2% of publications, cyst classification was not provided. In those publications where classification was conducted, 15% used the 'Gharbi's' classification and 15% used the WHO-IWGE classification (Tamarozzi et al., 2014a).

1.2.3.2 Imaging of abdominal cystic echinococcosis cysts

Radiography: In 20–30% of CE cases, calcification is observed on plain radiography. These calcifications typically have a ring-like or curvilinear pattern (Beggs, 1985; Pedrosa et al., 2000).

Ultrasonography: US is considered the gold standard imaging method (WHO-IWGE, 2003). The following stages are based on the WHO-IWGE classification.

Active stage CE1: In this early phase, CE may manifest as a well-defined anechoic cyst (active stage CE1). The cyst wall is usually observed as the double echogenic lines separated by a hypoechogenic layer termed the 'double contour sign' (Fig. 3A). No internal structures are observed in simple cysts. However, by repositioning the patient multiple echogenic foci, due to the presence of hydatid sand, may be detected within the lesion (Fig. 3B). The echogenic foci quickly fall to the lowest portion of the cavity without forming visible strata. This finding has been referred to as the 'snowstorm' or 'snowflakes sign'.

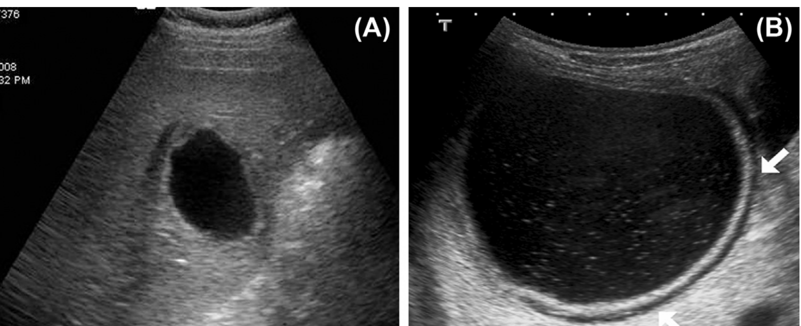


Figure 3 (A) Abdominal ultrasonography. WHO–IWGE CE1 lesion with well-defined contours and posterior acoustic enhancement. 'Double contour sign' can be seen as a layer of hyperechogenicity around the cystic lesion. (B) WHO–IWGE stage CE1 lesion with well-defined convex borders reflecting high intracystic pressure. Tiny innumerable echogenicity represents 'hydatid' sand inside CE lesion. Hyperechogenic line of 'double contour' is surrounded by hypoechoic rim secondary to albendazole treatment (*white arrows*).

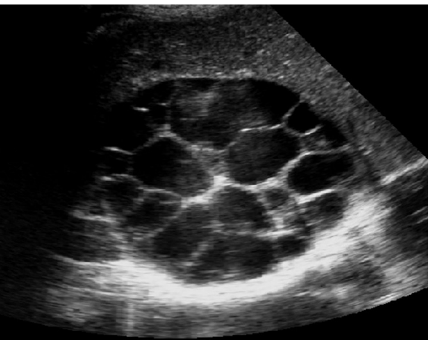


Figure 4 Abdominal ultrasonography. WHO-IWGE stage CE2 with multiple round-shaped aggregates belonging to daughter cysts. This appearance is called to be 'honeycomb pattern'.

Active stage CE2: These multiseptated cysts manifest as well-defined fluid collections in a 'honeycomb pattern', with multiple septa representing the walls of the parasitic vesicles. Separated, entire vesicles appear as 'cysts within a cyst' and are commonly referred to as 'daughter cysts' (Fig. 4).

Transitional stage CE3: These cysts have multiple internal septa, daughter cysts, multiple echogenic foci and floating membranes inside the cyst cavity. A decrease in intracystic pressure, cyst degeneration, trauma, host response or response to therapy may lead to detachment of the parasite from the host-derived adventitial layer (Fig. 5A).

Transitional stage CE3a: These cysts may appear as a well-defined collection of fluid, with a localized split in the wall and floating layers inside the cyst cavity. Complete detachment of the membranes into the cyst cavity is called as the 'US water-lily sign' (Fig. 5B).

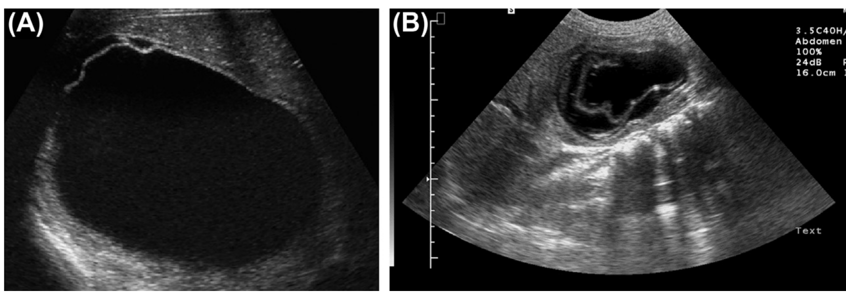


Figure 5 (A) Abdominal ultrasonography of cyst staged WHO-IWGE CE3a. Well defined linear echogenic line is visualized at the anterior aspect of the cystic lesion, representing detached membrane and detachment of parasitic layers (endocyst) from adventitia (pericyst). (B) Cyst shows completely detached membranes floating in the CE lesion (WHO-IWGE stage CE3a).

Transitional stage CE3b: In this stage, daughter cysts are separated by the echinococcal matrix (a material with mixed echogenicity) and demonstrate a 'wheel spoke' pattern (Fig. 6). The matrix represents echinococcal fluid containing membranes of broken vesicles, brood capsules, protoscoleces and 'hydatid sand'. Membranes may appear within the matrix as serpentine linear structures, a finding that is highly specific for diagnosis of CE. Presence of daughter cysts indicates the viability of the germinative layer.

Inactive stage CE4: In this stage the matrix fills the cyst completely, creating a mixed echogenic pattern that mimics a solid mass. This appearance is called as the 'ball of wool sign'. Because differentiation of this cyst type from other hepatic masses or abscesses is often difficult, it is important to look for membranes within the lesion that may help in making a correct diagnosis (Fig. 7A). While most CE4 cysts are inactive, the parasite may still be alive (Wang et al., 2006). Contrast-enhanced CT or MRI may be necessary to correctly identify this stage.

Inactive stage CE5: In this stage, calcification of cyst wall occurs. Calcification of the internal matrix may also be seen. These cysts have a hyperechoic contour, with a cone-shaped acoustic shadow. When the cyst wall is heavily calcified, only the anterior portion of the wall is visualized and appears as a thick arch with a posterior concavity which is an important sign for the diagnosis of inactive stage CE5 (Fig. 7B). Partial calcification of the cyst does not always indicate the death of the parasite; nevertheless, densely calcified cysts may be assumed to be inactive. CT or MRI can aid in the evaluation of heavily calcified cysts.

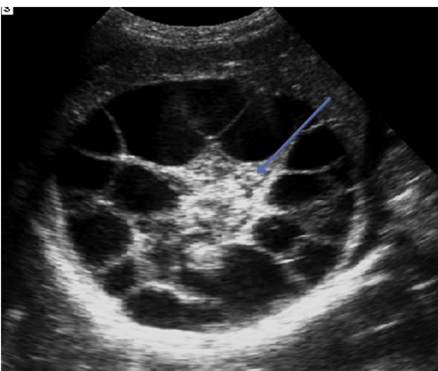


Figure 6 Abdominal ultrasonography of cyst staged WHO-IWGE CE3b. Presence of well-defined multiple daughter cysts separated by 'hydatid' matrix (*blue arrow*) which represents the 'wheel spoke' pattern of CE lesions.

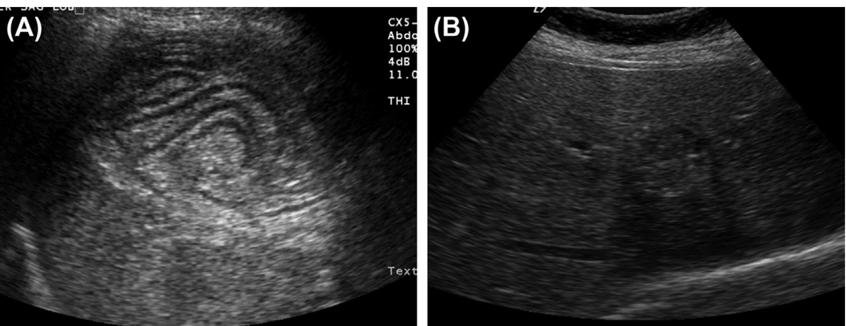


Figure 7 (A) Abdominal ultrasonography of cyst staged WHO-IWGE CE4. Heterogeneous lesion with mixed echogenicity. Detached membranes folding on itself is clearly visible. This pattern can be recognized as 'ball of wool sign'. (B) Abdominal ultrasonography shows internal and peripheral wall calcifications within the isoechogenic or mildly hyperechogenic lesion. Presence of such a totally calcified lesion indicates an 'inactive' lesion, WHO-IWGE stage CE5.

Computed tomography: While there is no cyst classification scale specific for CT, US classification methods may be used (Stojkovic et al., 2012). CT is actually the method of choice to study extrahepatic dissemination of cysts because it allows for imaging of the entire abdomen, pelvis and thorax (Fig. 8) (Oto et al., 1999). It is also commonly used in obese patients and those who have had previous abdominal surgery or who suffer from excessive intestinal gas. Common locations for extrahepatic dissemination of cysts

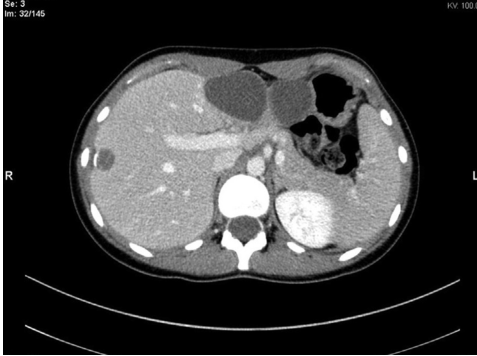


Figure 8 Postcontrast venous phase axial upper abdominal computed tomography image. Multiple cystic lesions in both hepatic lobes.

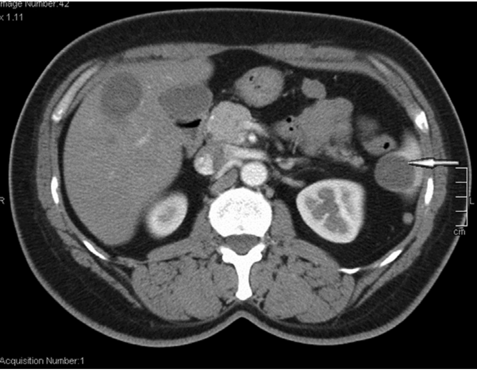


Figure 9 Postcontrast axial upper abdominal computed tomography image. Cystic lesions in right hepatic lobe and inferior portion of spleen (squiggly *white arrow*).

include the other abdominal organs, peritoneum, the diaphragm, the thoracic cavity, the abdominal wall, the portal system and other vessels (Chawla et al., 2003) (Fig. 9). Administration of intravenous (IV) contrast medium may be warranted to obtain a vascular map or if infection or communication with the biliary tree is suspected. Abscesses typically appear as a highly attenuated rim surrounding the lesion. Patchy areas of contrast-enhanced liver parenchyma, in the vicinity of the lesion, can represent inflammatory changes. Indirect signs of infection and/or communication with the biliary tree, including finding evidence of gas, air or gas inside the cyst (Fig. 10).

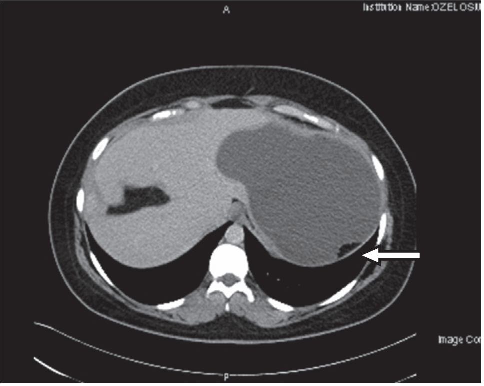


Figure 10 Postcontrast venous phase axial upper abdominal computed tomography image. A huge CE lesion is located in the left lobe of the liver containing a piece of fat (*Arrow*).

Calcification of the cyst wall can be detected by CT with completely calcified inactive CE5 cysts appearing as round hyperattenuating masses. However, CT has limited ability to evaluate internal septa, floating membranes and daughter cysts (Fig. 11). On CT, a CE cyst typically appears as a round lesion with water attenuation density, surrounded by a calcified ring-like or highly attenuated wall, representing the host-derived adventitial layer. Detachment of the parasite membranes from the adventitia is seen as linear areas of increased attenuation within the cyst. If daughter cysts are visualized, they typically contain fluid with a lower attenuation than the fluid in the main cyst. Positron-emission-tomography using ^{18}F-fluoro-desoxy-glucose as a tracer (FDG-PET), with or without CT, is not currently recommended since there is no FDG uptake except in CE cysts with bacterial infection (Niccoli Asabella et al., 2013).

Magnetic resonance imaging: MRI allows the visualization of cysts in multiple planes (Mendez et al., 1996; Marrone et al., 2012). It is also the best imaging modality detect biliary tree involvement MRI (Fig. 12). If US cannot be performed due to cyst location or patient-specific reasons, T2-weighted MRI is often preferable to CT except when it comes to evaluating calcifications (Stojkovic et al., 2012). MRI is the best diagnostic imaging modality to evaluate floating membranes and membrane detachment.

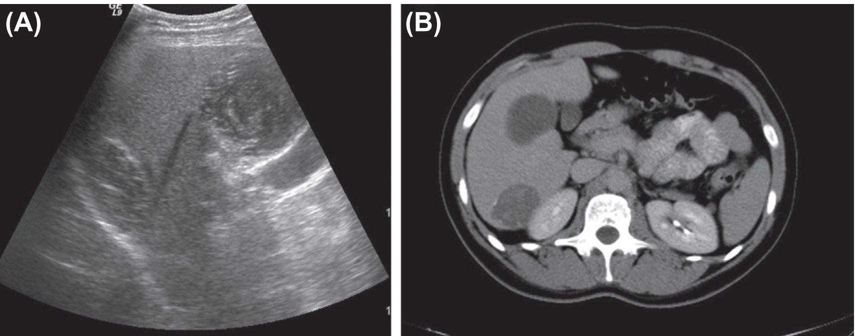


Figure 11 Abdominal ultrasonography (US) image (A) shows two solid-appearing lesions with mixed echogenicity. Internal heterogeneity of these cystic echinococcosis lesions is due to filling with 'hydatid' matrix. Apparent cystic component or daughter cyst cannot be distinguished by US (stage WHO—IWGE C4). Axial computed tomography (CT) image (B) shows the precise location, including the neighbouring anatomical structures, and the presence of internal/peripheral calcifications. A final diagnosis cannot be obtained by CT due to the combined cystic and solid character of the two CE lesions.

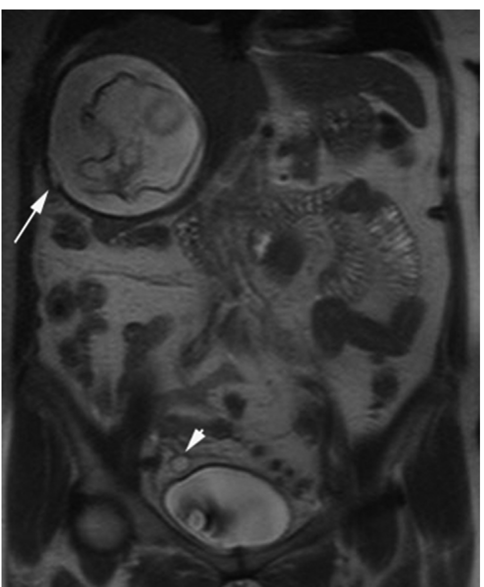


Figure 12 Coronal T2-weighted abdominal magnetic resonance image. Huge cystic lesion in the right hepatic lobe with intralesional linear, hypointense signals indicating detached membranes. There is a suspicious discontinuity (*white arrow*) in the posterior wall of the lesion. Small cystic lesion (*white arrowhead*), located in the superior neighbourhood of bladder, can be identified as a daughter cyst which dropped from the main lesion.

Diffusion-weighted MRI (DW-MRI) with a high b factor (1000 s/mm^2) is helpful in differentiating purely liquid cysts (active CE1) from benign liver cysts (CL). With this method, CE cysts appear hyperintense, whereas, benign cysts do not. In addition, using DW-MRI allows for the calculation of apparent-diffusion-coefficient (ADC) value, which can be used to assess the content of the hepatic lesions. The ADC of CE cysts tends to be very low due to the presence of protoscoleces, sodium chloride, proteins, glucose, ions, lipids and polysaccharides (Fig. 13) (Oruc et al., 2010; Inan et al., 2007).

Although CE cyst fluid tends to appear hypointense on T1-weighted and hyperintense on T2-weighted MR images, heterogeneous signal intensity on T2-weighted images is not uncommon (Fig. 14). The cyst's host-derived adventitial layer that usually appears as a low-signal-intensity rim on T2-weighted images (Erdem et al., 2014). In addition, there may be an intermediate-signal-intensity inner ring representing the detached parasitic layers. Adventitial layers may show slight enhancement after IV

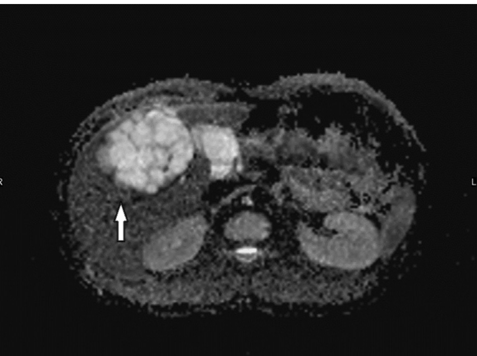


Figure 13 Apparent-Diffusion-Coefficient (ADC) map constructed from diffusion weighted images of upper abdomen shows multicystic lesion (*white arrow*) in right hepatic lobe. An ADC value of the lesion is slightly lower than simple hepatic cysts due to presence of 'hydatid' matrix.

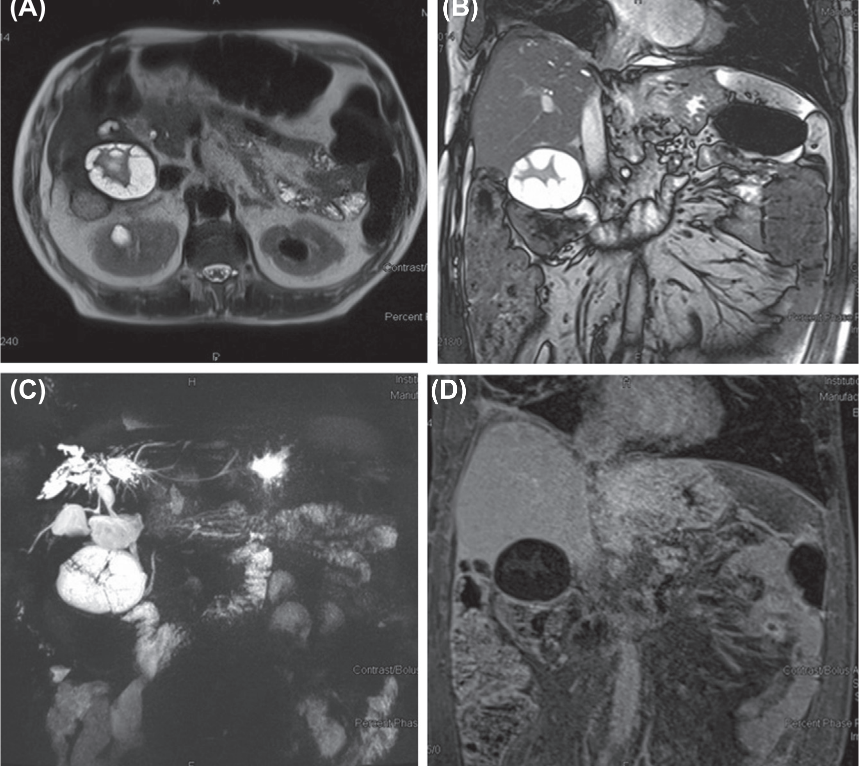


Figure 14 Axial (A) and coronal (B) T2-weighted upper abdominal magnetic resonance imaging. WHO–IWGE CE 3b cyst. Daughter cysts and fluid components are hyperintense, in contrast 'hydatid' matrix appears hypointense in central location. Coronal maximum intensity projection magnetic-resonance-cholangiopancreaticography (C) depicts CE3b lesion and peripheral intrahepatic biliary ducts. On postcontrast T1-weighted magnetic resonance imaging (D) cystic parts do not enhance.

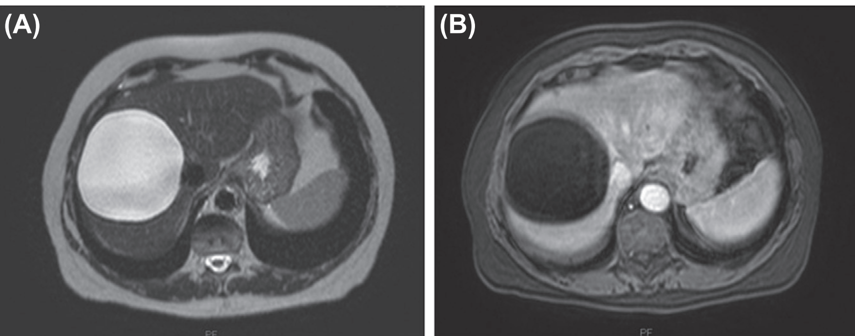


Figure 15 Axial T2-weighted magnetic resonance imaging. (A) The hyperintense cystic lesion (WHO–IWGE CE 1) in the right hepatic lobe is surrounded by a hypointense rim. Axial postcontrast T1-weighted magnetic resonance imaging (B) reveals slight contrast enhancement of adventitia (pericyst).

injection of gadolinium as a contrast agent (Fig. 15). The 'snake sign' is typical MRI imaging feature depicting collapsed parasitic membranes, secondary to damage or degeneration of the cyst. These membranes have low signal intensity. The intracystic air–fluid level may be visible on MRI, as a possible sign of infection (Marrone et al., 2012).

Magnetic resonance cholangiopancreatography (MRCP): This method enhances visualization of communication between the CE cyst and biliary tree and dilatation of the biliary system secondary to compression of the cyst (Fig. 16) (Mendez et al., 1996; Hosch et al., 2007).

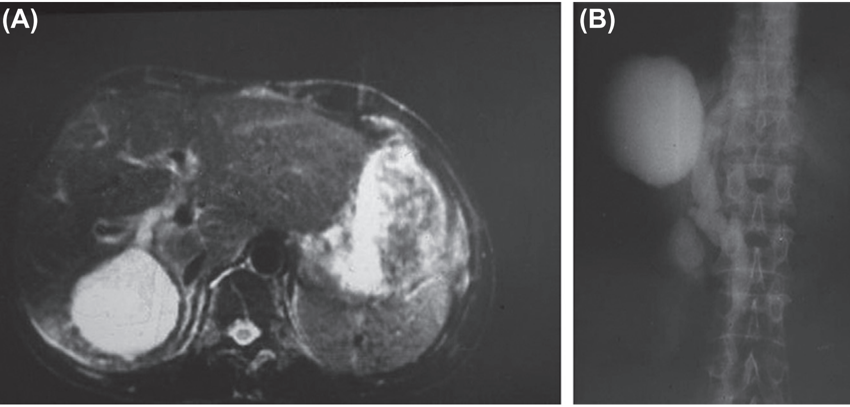


Figure 16 Axial fat saturated T2-weighted magnetic resonance imaging of upper abdomen. (A) CE lesion located in the posterior segment of the right hepatic lobe communicating with the biliary tree. Cystography under fluoroscopy (B) identifies the cystic lesion with biliary intrahepatic ductal filling.

Proton magnetic resonance spectroscopy (^{1}H-MRS): ^{1}H-MRS provides additional information based on the metabolic composition of the CE cysts (Barker, 2005; Limanond et al., 2004). This method may aid in determining the viability of the GL and protoscoleces (Hosch et al., 2008). Currently, ^{1}H-MRS is mainly used in the evaluation of brain lesions due to the presence of motion-related artefacts associated with hepatic cysts.

1.2.3.3 Imaging of thoracic cystic echinococcosis cysts

Thoracic radiographs (chest X-rays) and CT scans are the most important imaging modalities for the diagnosis of pulmonary CE. Uncomplicated cysts are visualized as round or oval masses with well-defined borders. Complicated cysts may be diagnosed by commonly accepted pathognomonic signs that are discussed below.

Chest X-ray: Pulmonary cysts can range from 1 to 20 cm in diameter (Pedrosa et al., 2000). On chest X-ray, intact cysts typically appear as homogeneous round or oval-shaped structures with smooth borders surrounded by normal lung tissue. Large cysts can shift the mediastinum, induce a pleural reaction or cause atelectasis of the adjacent parenchyma (Fig. 17). Cyst growth produces erosions in the bronchioles and, as a result, air is introduced between the adventitia and LL, producing the 'crescent' or 'meniscus' sign. Some consider this to be a sign of impending cyst rupture. Air penetrating the interior of the cyst may outline the inner surface of the LL, producing parallel arches of air that are referred to as 'Cumbo's sign'. This phenomenon has also been described as having an onion peel appearance (Morar and Feldman, 2003). If a ruptured cyst communicates with the tracheobronchial tree, evacuation of the cyst contents results in an air/fluid

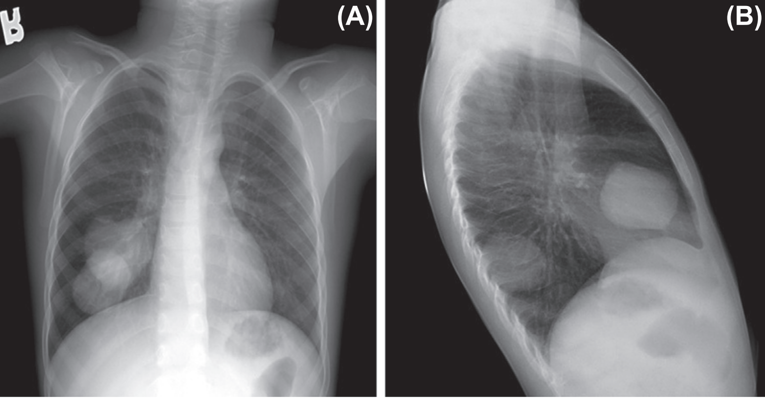


Figure 17 Posteroanterior (A) and lateral (B) chest radiographs. Two well-defined radioopacities can be visualized in right lung. Further diagnostic work-up revealed pulmonary stage WHO—IWGE CE1.

interface. After partial expectoration of the cyst fluid and protoscoleces the cyst empties and the collapsed membranes can be seen inside the cyst producing the 'serpent sign'. When the crumpled endocyst floats freely in the cyst fluid, it is known as the 'water-lily sign' (Fig. 18). In a minority of cases, when the fluid is completely evacuated by expectoration, the remaining solid components fall to the lower part of the cavity, with the resulting mass called 'Monod's sign'.

Ultrasonography: US is typically not indicated for pulmonary CE unless the cysts are close to the pleural surface (Zeyhle, personal communication). However, it is important to note that abdominal US may reveal concomitant liver involvement in up to 15% of patients with pulmonary CE. While there is no staging scale specific for pulmonary cysts the WHO-IWGE classification of liver cysts may be applied (Akhan, personal communication).

Computed tomography: CT is an important imaging modality to diagnose complications associated with pulmonary CE (Fig. 19). If the cyst is intact, contrast-enhanced CT may show a thin enhancing rim. The contents of these cysts appear homogeneous, with a density close to that of water [1−10 Hounsfield units (HU)]. Unruptured cysts are often indistinguishable from a variety of other pulmonary lesions. However, the presence of daughter cysts is helpful in making a diagnosis. The 'inverse crescent sign' results from the separation of the membranes from the posterior side of the cyst without any anterior extension. This is due to blebs of air dissecting the wall of the cyst, which appears ring-shaped ('signet ring sign'). The 'air bubble sign' is present when air dissection occurs between the adventitia and the parasitic layers due to erosion of a bronchiole by the expanding cyst. This sign is best seen in the mediastinal window as single or multiple, small, rounded

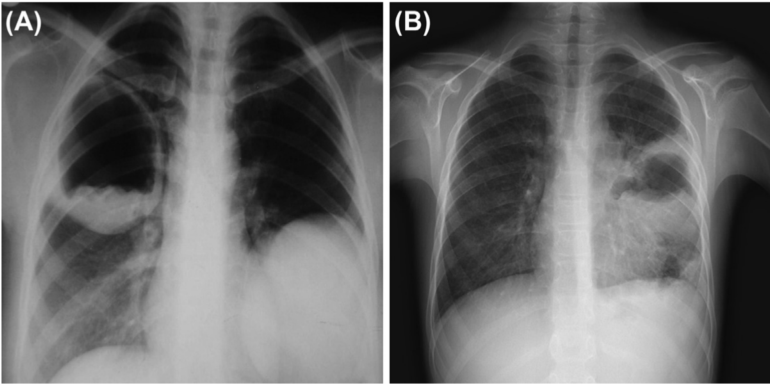


Figure 18 Posteroanterior (PA) chest radiograph (A) shows a cystic lesion in middle zone of right lung. Radio-opacity in the lower part of the cystic lesion represents floating detached membranes ('water-lily sign'). (B) PA radiograph from another patient with cystic lesion and undulating membrane in midlower zones of the left lung.

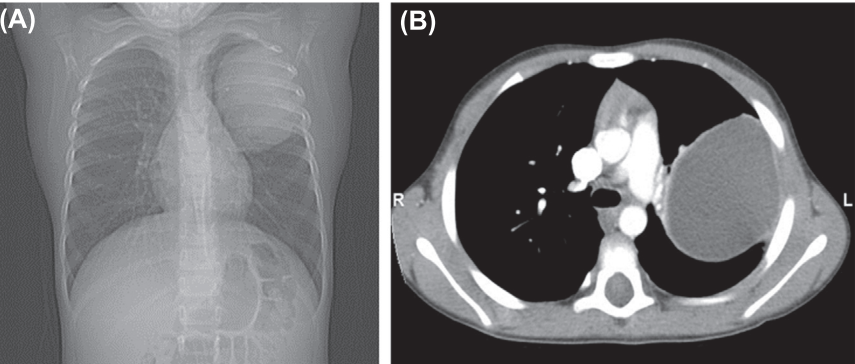


Figure 19 Scout image of thoracic computed tomography (CT) (A) shows a mass with well-defined borders extending through mid to upper zones of left lung. Axial postcontrast thorax CT section (B) reveals a purely cystic lesion in left lung when using a soft tissue window.

radiolucent areas with sharp margins at the periphery of a solid mass. After the injection of contrast medium, infected cysts appear as poorly defined masses with an increased density and contrast enhancement around the cyst wall ('ring enhancement sign') (Kervancioglu et al., 1999).

Magnetic resonance imaging: The MRI characteristics of a pulmonary cyst may differ depending on the cyst's developmental phase, whether the cyst is uni- or multiseptated, and whether the cyst is viable, infected or dead. MRI can also be used to assess reactive changes in the host tissue. With MRI, pulmonary cysts show low signal intensity on T1-weighted images and high signal intensity on T2-weighted images (Figs. 20 and 21).

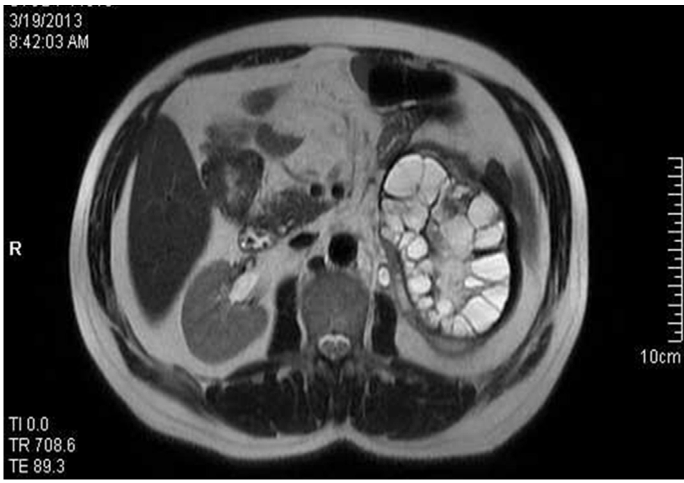


Figure 20 Axial T2-weighted magnetic resonance imaging of upper abdomen. Multicystic, heterogeneous lesion in the kidney, which was a 'confirmed' CE lesion with multiple daughter cysts (WHO—IWGE stage CE3b).

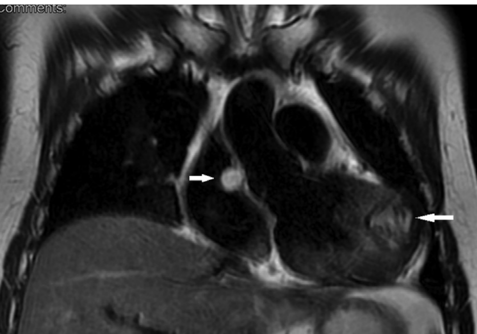


Figure 21 Coronal thoracic magnetic resonance image. Two cystic lesions located in the heart (*arrows*).

1.2.3.4 Imaging of cystic echinococcosis brain and bone cysts

Brain: On CT scans, intraparenchymal CE1cysts are usually well-defined, thin-walled and spherical. These cysts contain fluid that appears to have the same density as cerebrospinal fluid (Khaldi et al., 2000). Generally, brain cysts do not show contrast enhancement or perilesional oedema (Fig. 22). However, large cyst may produce a mass effect resulting in ventricular compression (Stojkovic and Junghanss, 2013). Intracystic calcifications and calcifications around the cyst margins are rare (Altinörs et al., 2000).

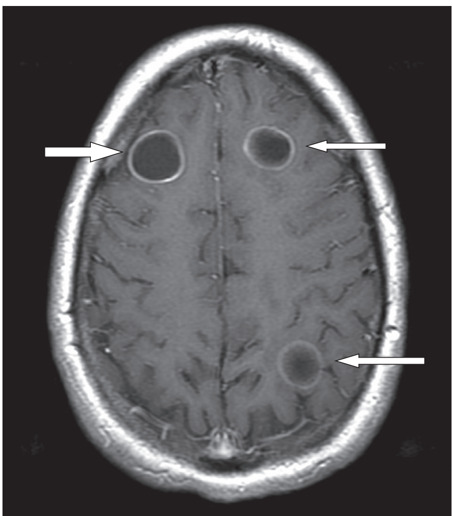


Figure 22 Brain magnetic resonance imaging. Three cystic lesions (*arrows*) are shown. The WHO—IWGE CE1 lesions are surrounded by two layers; inner layer appears hyperintense, and the thin outer layer is hypointense.

MRI is the method of choice for the diagnosis of brain CE, where T2-weighted images show a characteristic low signal intensity rim around the cyst. ^{1}H-MR spectroscopy imaging has been used to produce quantitative metabolic profiles of brain CE fluid and to differentiate cyst stages (Seckin et al., 2008; Hosch et al., 2008). An example of the differential diagnosis of a brain abscess is displayed in Figs. 23A–F.

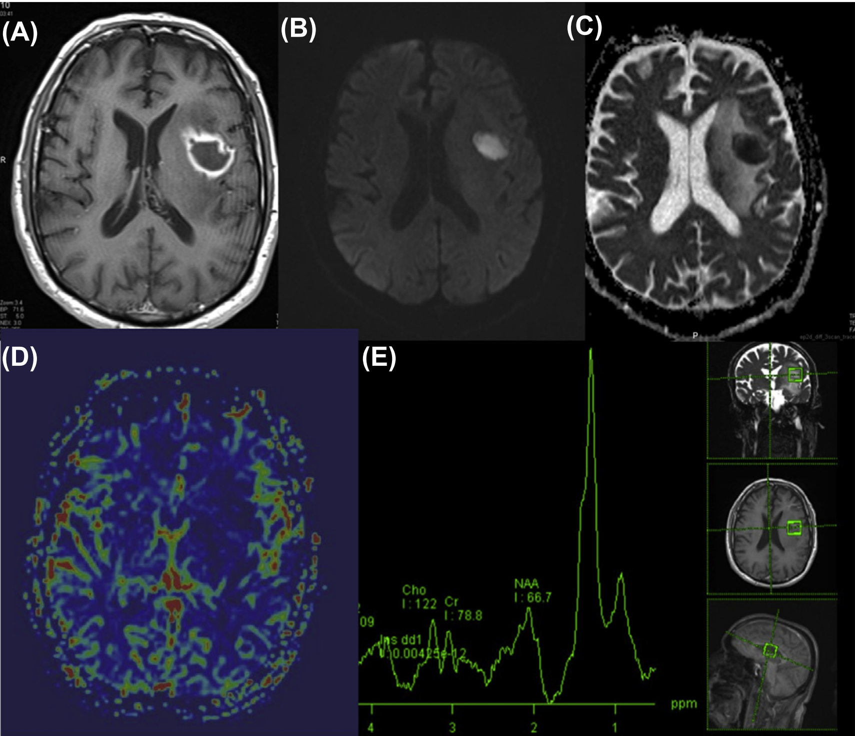


Figure 23 Brain magnetic resonance imaging (postcontrast T1 weighted imaging; (A) along with diffusion (B, C) and perfusion (D) weighted imaging, single voxel proton magnetic resonance spectroscopy (MRS) obtained at a short echo time (TE:35 msn) performed in a 73-year-old man presented with a mass in the left insula. The mass in the left insula has a marked accompanying oedema and irregular peripheral enhancement (A). There is restricted diffusion in the mass as revealed by marked hyperintensity on diffusion imaging (B) and very low apparent diffusion coefficient (ADC) values on corresponding ADC map (C). Cerebral blood volume map derived from dynamic T2* weighted perfusion imaging shows very low CBV (D) and MRS shows suppressed N-acetyl aspartate (NAA), choline (Cho) and creatine (Cr), but markedly high lactate and aminoacids (E). Marked diffusion restriction, hypoperfusion and very low neuroaxonal markers (probably due to partial inclusion of surrounding parenchyma into the MRS voxel) in absence of elevated choline all suggested diagnosis of brain abcess for this irregularly enhancing mass with profound edema in this patient without a systemic symptom. Pathological and microbial examination was consistent with the bacterial abscess. *Courtesy by Kader Karlı Oguz.*

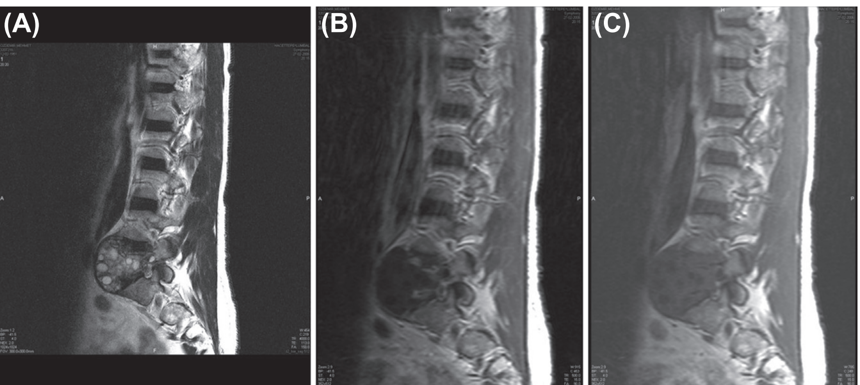


Figure 24 Sagittal T2-weighted, pre- and postcontrast T1-weighted magnetic resonance imaging (A–C) of lumbosacral spine show a heterogeneous, multicystic lesion predominantly involving body of L5 vertebra. There is accompanying minimal height loss in involved vertebral body and anterior component of lesion extends to prevertebral tissues. Well-defined cystic components are hypointense on T1-weighted images whereas hyperintense on T2-weighted images and do not exhibit contrast enhancement. *Courtesy by Üstün Aydıngöz.*

Bone: Diagnosis of bone CE is primarily based on plain radiography (Zlitni et al., 2001). X-rays may show the actual destruction of the surrounding bone, making it difficult to differentiate from bone tumours. In cases with spinal CE, destruction of vertebral bodies and intervertebral space narrowing can be seen. On CT scans, bone cysts typically appear as round space-occupying lesions, with double layered arcuate calcification (Fig. 24). CT images can detect a ruptured cyst by showing detached layers. Overall, MRI is the most helpful method for diagnosing bone CE due to better visualization of the cyst in relation to surrounding tissue (Singh et al., 1998).

1.2.3.5 Differential diagnosis

The differential diagnosis for CE depends on the cyst characteristics (e.g., number, dimension, stage) and the organ where the cyst is located. Cystic liver lesions are commonly encountered in practice, and may be classified into four categories as congenital or developmental, neoplastic, inflammatory or miscellaneous (Akhan et al., 1994a; Zeitoun et al., 2004; Bracantelli et al., 2005; Akinci et al., 2005; Akhan et al., 2007b, Akhan and Koroglu (2007); Czermak et al., 2008).

Cystic lesions (WHO—Informal Working Group on Echinococcosis classification): CLs can be solitary or multiple and are typically 30—50 mm in diameter. These cysts may eventually be reclassified as CE1 after completion of additional diagnostic testing (Brunetti et al., 2010). The most important imaging feature to differentiate CL from CE1 is the 'double contour' sign which is pathognomonic sign for CE (Fig. 25).

Solitary simple liver cyst: These cysts have a lower internal pressure than CE cysts and appear to occur more frequently in middle-aged and older females. On US examination, simple cysts are anechoic with sharp margins and posterior acoustic enhancement. The cyst wall appears thin and hyperechoic. On CT examination a simple cyst appears as a homogeneous lesion with water-like density that does not show contrast enhancement. These cysts are hyperintense on T2-weighted MR images and hypointense on T1-weighted MR images.

Polycystic liver disease: Polycystic liver disease occurs more frequently in females. The lesions can be seen in one lobe or throughout both liver lobes. In more than 50% of cases the disease is associated with polycystic kidneys (Fig. 26). US, CT and MRI show multiple, simple hepatic CLs without enhancement after contrast. Polycystic liver disease rarely resembles the honeycomb appearance that is typical for CE2 cysts.

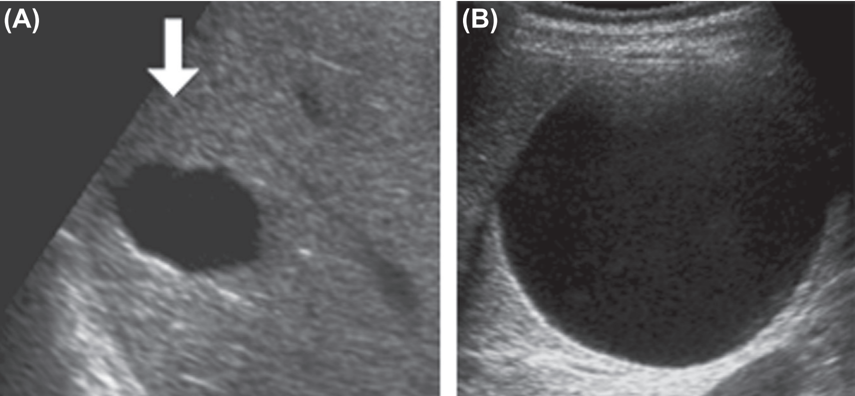


Figure 25 Abdominal ultrasonography in two different patients. Simple liver cyst (A) and cystic echinococcosis cyst (B). Presence of concave and lobulated borders (*white arrow*), thin, imperceptible wall with posterior acoustic enhancement is typical for simple liver cysts (A). Prominent convex borders, reflecting high intracystic pressure with accompanying double echogenicity ('double contour sign') makes the diagnosis of liver cystic echinococcosis likely (B).

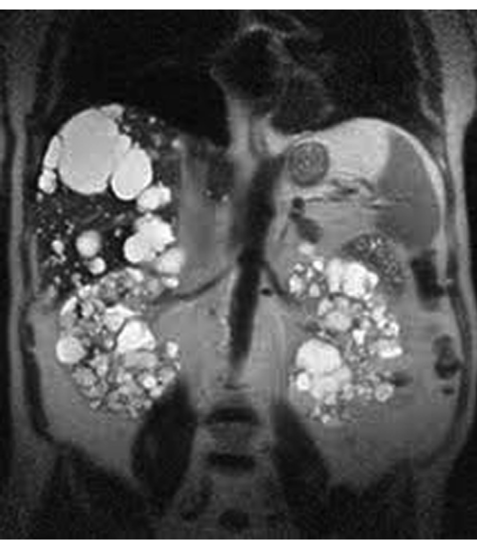


Figure 26 Coronal T2-weighted magnetic resonance imaging. Multiple T2-hyperintense cystic lesions in bilateral kidneys as well as in liver. The findings are compatible with autosomal dominant polycystic kidney disease and polycystic hepatic disease.

In Caroli's disease, cysts emerge from saccular dilation of large intrahepatic bile ducts. The 'central dot sign' represents a portal branch located within the cyst. This sign is pathognomonic for Caroli's disease and excludes the presence of CE1 cysts (Yüce et al., 2002) (Fig. 27).

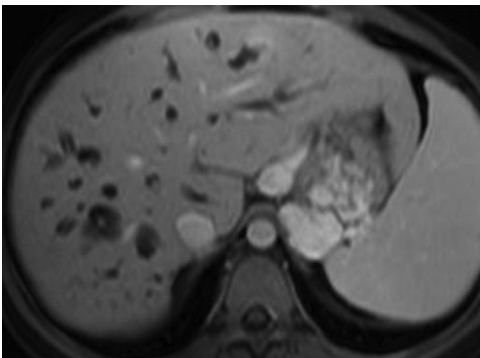


Figure 27 Postcontrast, transverse T1-weighted fat-saturated magnetic resonance imaging. Multiple, hypointense cystic structures in both lobes of the liver. Central portal radicles with contrast enhancement forms the typical 'central dot' sign of Caroli's disease. Presence of multiple varicose veins next to the splenic hilum suggests portal hypertension.

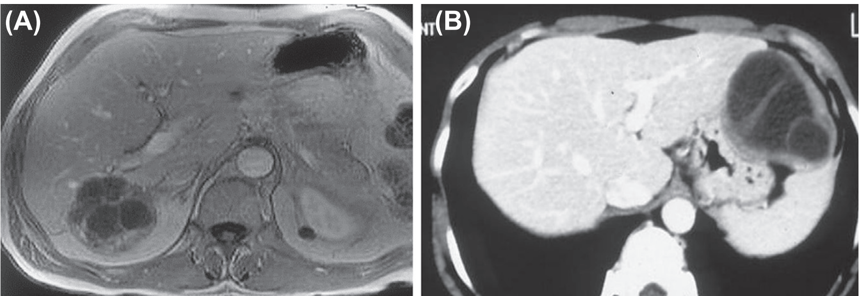


Figure 28 Postcontrast transverse T1-weighted MR image (A) and postcontrast transverse venous phase computed tomography image (B) of upper abdomen in two different patients. Multicystic, lobulated lesions in liver. Presence of enhancing septae favours presumptive diagnosis of biliary cystadenoma/cystadenocarcinoma (A). In liver cystic echinococcosis, there is no enhancement of septae (B).

Neoplastic cysts: Cystadenomas occur more frequently in middle-aged patients. They are rare and slow-growing tumours arising from embryonic or ectopic bile ducts. Biliary cystadenocarcinoma usually results from malignant transformation, but can also occur de novo. These lesions are encapsulated and contain mucoid material (Agildere et al., 1991). On US examination the cysts appear multiseptate with thin internal walls. They may present with calcifications and/or mural nodules. Enhancement of septa can be seen on Doppler US. In contrast to cystadenoma or cystadenocarcinoma, septae in CE2/CE3b cysts are never enhanced after IV contrast is administered (Koroglu et al., 2006) (Fig. 28).

1.2.4 Confirmation of diagnosis

As outlined in Table 1, clinical experts associated with the WHO-IWGE recommended that CE cases be described as 'confirmed', 'probable' or 'possible' based on available diagnostic methods and findings (Brunetti et al., 2010). Adjunctive methods, such as the detection of serum antibodies against the parasite, can aid in moving closer to a definite diagnosis. Serological assessment typically follows a two-step approach (Siles-Lucas and Gottstein, 2001; for details see Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017). In the first step, diagnostically sensitive tests are employed [e.g., indirect haemagglutination test, IHA, or enzyme-linked immunosorbent assay (ELISA)] using *E. granulosus* crude antigens. However, these tests lack specificity and cross react with other helminthic infections and also gastrointestinal malignancies. In a second step a highly specific test is used

Table 1 Diagnosis criteria of cystic echinococcosis (CE)

A) Clinical criteria	**1.** A slowly growing tumour or static cystic mass (signs and symptoms vary with cyst location, size, type and number) diagnosed by imaging techniques. **2.** Anaphylactic reactions due to ruptured or leaking cysts. **3.** Incidental finding of a cyst by imaging techniques in asymptomatic carriers or detected by screening strategies.
B) Diagnostic criteria	**1.** Typical organ lesion(s) detected by imaging techniques (e.g., US, CT, radiography, MRI). **2.** Specific serum antibodies assessed by high-sensitivity serological tests, confirmed by a separate high specificity serological test. **3.** Histopathology or parasitology compatible with cystic echinococcosis (e.g., direct visualization of the protoscoleces or hooklets in cyst fluid). **4.** Detection of pathognomonic macroscopic morphology of cyst(s) in surgical specimens.
C) Case definition and likelihood of CE diagnosis	'Possible' case: Any patient with a clinical or epidemiological history and imaging findings or serology positive for CE. 'Probable' case: Any patient with the combination of clinical history, epidemiological history, imaging findings and serology positive for CE on two tests. 'Confirmed' case: The above, plus either (1) demonstration of protoscoleces or their components, using direct microscopy or molecular tools, in the cyst contents aspirated by percutaneous puncture or at surgery or (2) changes in US appearance, e. g., detachment of the parasitic cyst in a CE1 cyst, thus moving to a CE3a stage, or solidification of a CE2 or CE3b, thus changing to a CE4 stage, after administration of benzimidazoles (at least 3 months) or spontaneously.

CT, computed tomography; *MRI*, magnetic resonance imaging; *US*, ultrasound; CE1, CE2, CE3a, CE3b and CE4 refer to the WHO−IWGE ultrasound classification of hepatic CE cysts (WHO-Informal Working Group on Echinococcosis, 2003; Brunetti et al., 2010).

to confirm the results of the screening tests. Many factors may influence test results, including the age and stage of the cyst, whether or not the cyst is intact and whether or not extrahepatic cysts are present. Local infection pressure may influence test results, with children in highly endemic areas found to be seropositive in the absence of detectable cysts (Yang et al., 2008).

A 'confirmed' diagnosis of CE (Table 1) can be achieved by identifying a pathognomonic sign during diagnostic intervention or by parasitological diagnosis. At the initial puncture (technical details see Annex 1), when the needle is removed, the crystal-clear fluid gushes out as a result of high pressure inside of the cyst, and this is an accepted pathognomonic criterion for the viability of the parasite (Akhan et al., 1996). The pressure may be >35 cm of water, in contrast to a lower pressure for benign cysts or 'inactive' CE stage cysts (CE4 or CE5) (Yalin et al., 1992). Another criterion is the separation of the parasite layers from adventitia during the procedure, as shown in Fig. 29A and B.

Microscopic examination of the cyst fluid may reveal motile protoscoleces or only hooklets (Smyth and Barrett, 1980). A diagnosis of CE can also be confirmed by histopathology. Presently a species-specific antibody staining of the parasite wall is currently not available for the diagnosis of CE as it is for alveolar echinococcosis (AE) (Barth et al., 2012). Molecular diagnosis (i.e., detection of *Echinococcus* spp. antigens and/or DNA) and species differentiation can be achieved in specialized laboratories (for details see Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017).

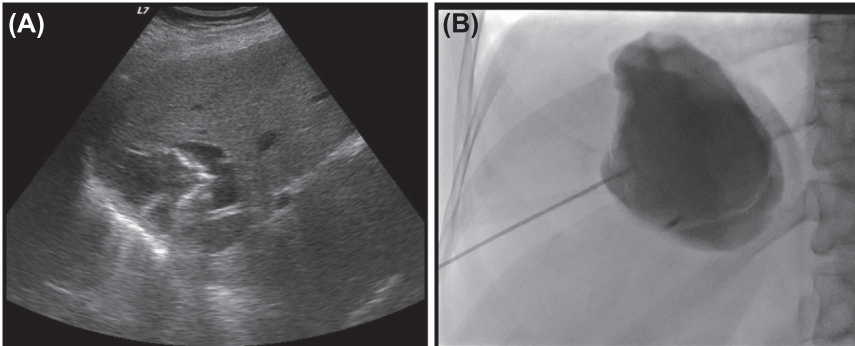


Figure 29 Ultrasonography image (A) and cavitography (B) show detachment of parasitic layers from the host adventitia within several minutes after the puncture.

1.3 Clinical management

1.3.1 Rationale and rules for a stage-based therapeutic strategy

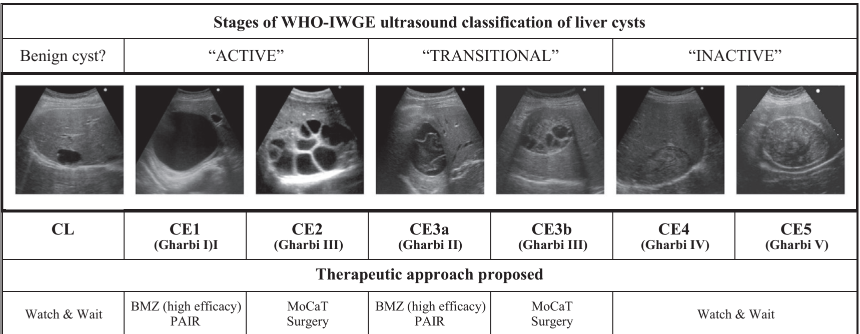
Treatment of uncomplicated CE should be based on cyst localization, diagnostic imaging features, available medical/surgical expertise and equipment, and the likelihood of patients to adhere to long-term monitoring. Because the treatment involves a variety of options and requires specific clinical expertise, patients should be referred to recognize national/regional CE treatment centres, whenever possible (Brunetti et al., 2010). Treatment of CE aims to destroy the metacestode, and this can be achieved by sterilization of the parasite contents and removal of the entire fluid and parts of the parasite by aspiration or by surgical excision of the entire cyst (Sayek and Onat, 2001; Buttenschoen and Carli Buttenschoen, 2003; Menezes da Silva, 2003; Junghanss et al., 2008; Dziri et al., 2009). Sterilization of the cyst can be achieved by using scolecidal solutions, such as hypertonic saline (30%) or absolute alcohol (95%), injected into the cyst and/or by oral medication with BMZ carbamates , namely albendazole (ABZ) or mebendazole (MBZ) (Teggi et al., 1993; Keshmiri et al., 2001). This is followed by an involution process during which the parasite is gradually dying off leaving behind a solidified, often calcified cyst or a scar.

For patients with uncomplicated liver and/or abdominal cysts, four principal approaches are applied: (1) drug treatment with a BMZ; (2) percutaneous sterilization techniques; (3) minimally invasive or general surgery and (4) watch-and-wait. These approaches can be used independently or in combination. When performing surgery or cyst puncture, clinicians should be aware of the risk of anaphylaxis and prepared to address this possible complication. Currently, treatment/cyst management recommendations are specified for the following ultrasound defined WHO-IWGE types: CL, CE1, CE2, CE3a, CE3b, CE4 or CE5. The preferential use of the various therapeutic approaches is schematically highlighted in Fig. 30.

1.3.2 Treatment of abdominal cystic echinococcosis cysts

1.3.2.1 Drug therapy

MBZ was the first BMZ found to have in vivo activity against CE (Schantz et al., 1982). Presently, *ABZ* is the drug of choice for treating CE, as its bioavailability, albeit poor, is better than that of other antiinfective agents



Stages of WHO-IWGE ultrasound classification of liver cysts						
Benign cyst?	"ACTIVE"		"TRANSITIONAL"		"INACTIVE"	
CL	**CE1 (Gharbi I)I**	**CE2 (Gharbi III)**	**CE3a (Gharbi II)**	**CE3b (Gharbi III)**	**CE4 (Gharbi IV)**	**CE5 (Gharbi V)**
Therapeutic approach proposed						
Watch & Wait	BMZ (high efficacy) PAIR	MoCaT Surgery	BMZ (high efficacy) PAIR	MoCaT Surgery	Watch & Wait	

Figure 30 Stage-specific treatment of uncomplicated cystic echinococcosis in relation to the WHO—IWGE and 'Gharbi's' ultrasound classification of liver cysts. *BMZ*, benzimidazole carbamates; *MoCaT*, modified catheterization technique; *PAIR*, puncture-aspiration-injection-reaspiration. *Figures within the table are provided by E. Brunetti*

that have been utilized. ABZ has a half-life of 8.5 h and, for the treatment of CE, is typically administered b.i.d. to a total daily dose of 10—15 mg/kg/day (Horton, 1997). Alternatively, MBZ may be used t.i.d. to a total daily dose of 40—50 mg/kg body weight. To increase intestinal absorption, both drugs need to be taken with fatty foods (Brunetti et al., 2010). Duration of treatment depends on the individual situation, stage and size of the CE cyst, and patients should be monitored with imaging. Current recommendations suggest continuous treatment for several months. The previously recommended 'cyclic administration', with treatment interruption, should be avoided (Brunetti et al., 2010).

Hepatic and haematologic toxicities are the most frequent serious adverse effects associated with BMZ administration. For patients receiving BMZ therapy, it is generally recommended to have liver enzymes and complete blood cell counts monitored every two weeks during the first months of drug treatment. BMZs must be used with caution in patients with chronic hepatic disease and avoided in those with bone marrow depression. Alopecia is a recognized side effect in patients with chronic cholestasis and/or portal hypertension (Brunetti et al., 2010). The BMZ compounds are contraindicated for treatment of cysts at risk of rupture. They are also contraindicated in woman during early pregnancy, since they have been shown to be teratogenic in rats and rabbits (Bradley and Horton, 2001).

Benzimidazoles without additional interventional procedures: The impact of treatment solely with a BMZ depends on the stage of the cyst and on the cyst's GL integrity. BMZs are most effective on young cysts (e.g., CE1). In contrast, effectiveness on CE2 cysts is less than 50%. This class of drugs is also more effective on liver cysts than on cysts in other locations, presumably because the drugs can reach a higher concentration in the liver compared to other organ systems. Small cysts (<5 to 6 cm) CE1 and CE3a cysts located in the liver and lungs may respond favourably to sole treatment with a BMZ (Stojkovic et al., 2009; Salinas et al., 2011). Drugs alone are not effective against giant cysts (>10 cm in diameter). Sole treatment with BMZ is also indicated for patients with inoperable liver or lung CE; patients with multiple cysts in two or more organs and patients with peritoneal cysts.

Benzimidazoles with additional interventional procedures: BMZs are also used as an adjunct to surgery or interventional procedures to reduce the cyst's internal tension, to complement the mechanical removal of the cyst or the chemical sterilization of the parasite and to prevent secondary echinococcosis (Khuroo et al., 1993; Gil-Grande et al., 1993; Brunetti et al., 2010). A prospective study demonstrated that a protocol that combines ABZ and PAIR reduces the chance of cyst recurrence (Akhan et al., 2014). However, the most effective duration of BMZ administration before and after PAIR has not been well established. CE treatment centres recommend combined pre- and postoperative ABZ use between one and four months (Akhan, personal communication). Akhan et al. (2014) suggested that longer post-PAIR treatment may be associated with a higher frequency of ABZ-associated side effects.

At present, surgeons tend to administer ABZ from one week to one day before and from one to three months after intervention. Actual duration of treatment is dependent on surgical factors such as whether or not the cyst is opened. ABZ treatment is typically administered for one month after surgery in patients who have successfully undergone complete surgical resection of the cyst (radical procedure) or PAIR. The recommended treatment time extends to 3–6 months in patients with incompletely resected cysts (nonradical procedures), or when spillage has occurred during surgery or PAIR (Arif et al., 2008).

Use of praziquantel (PZQ): PZQ has been shown to be an effective scolecide in vitro, and in animal models (Morris et al., 1990). The drug,

an isoquinolone derivative, increases the permeability of the parasite's cell membrane to calcium, resulting in strong contractions and paralysis of the musculature leading to detachment from host tissue. The drug is not parasitocidal for *Echinococcus* spp. metacestode cysts, but can increase the bioavailability of BMZ when given together (Homeida et al., 1994; Cobo et al., 1998; Na-Bangchang et al., 2006; Garcia et al., 2016). Although PZQ is often used to prevent secondary echinococcosis, prospective studies are needed to determine the effectiveness of the drug in this capacity (Bygott and Chiodini, 2009).

1.3.2.2 Interventions that inactivate, remove or destroy parasitic tissue

The inactivation of the parasite is achieved by sterilization of the cyst content using a scolicidal solution injected into the cyst cavity (reviewed by Tamarozzi et al., 2014b). After the injection the cyst's liquid contents together with the parasite membranes is removed. Herewith, the viable metacestode is likely to be destroyed. All these interventions are performed by percutaneous approaches (Fig. 31). There are three techniques for the percutaneous treatment of CE cysts: (1) puncture of the cyst, aspiration of the cyst's content, injection of hypertonic saline solution or alcohol and reaspiration of fluid, known as 'PAIR' (2) the standard catheterization technique and (3) the modified catheterization technique (MoCaT). Techniques one and two sterilize the cyst using chemical agents, which is then usually accompanied by treatment with BMZ. Technique 3 differs in that, in addition to sterilization procedures, the parasitic membranes are also removed.

PAIR technique (Ben-Amor et al., 1986; Filice et al., 1990; WHO-IWGE booklet, 2001) (Annex 1): PAIR is the preferred treatment for WHO-IWGE type CE1 and CE3a hepatic cysts under 10 cm in diameter. Compared to traditional surgery, this technique is less invasive, less painful, less expensive and has a lower complication rate with earlier discharge from the hospital and return to normal activities (Akhan et al., 1996; Koroglu et al., 2014). If a cysto-biliary fistula is detected during the procedure, or any technical problem is occurring, the intervention can be successfully completed using the standard catheterization technique.

Standard catheterization technique (Akhan et al., 1993, 1996; Ustünsöz et al., 1999) (Annex 1): This technique is an alternative to PAIR and can

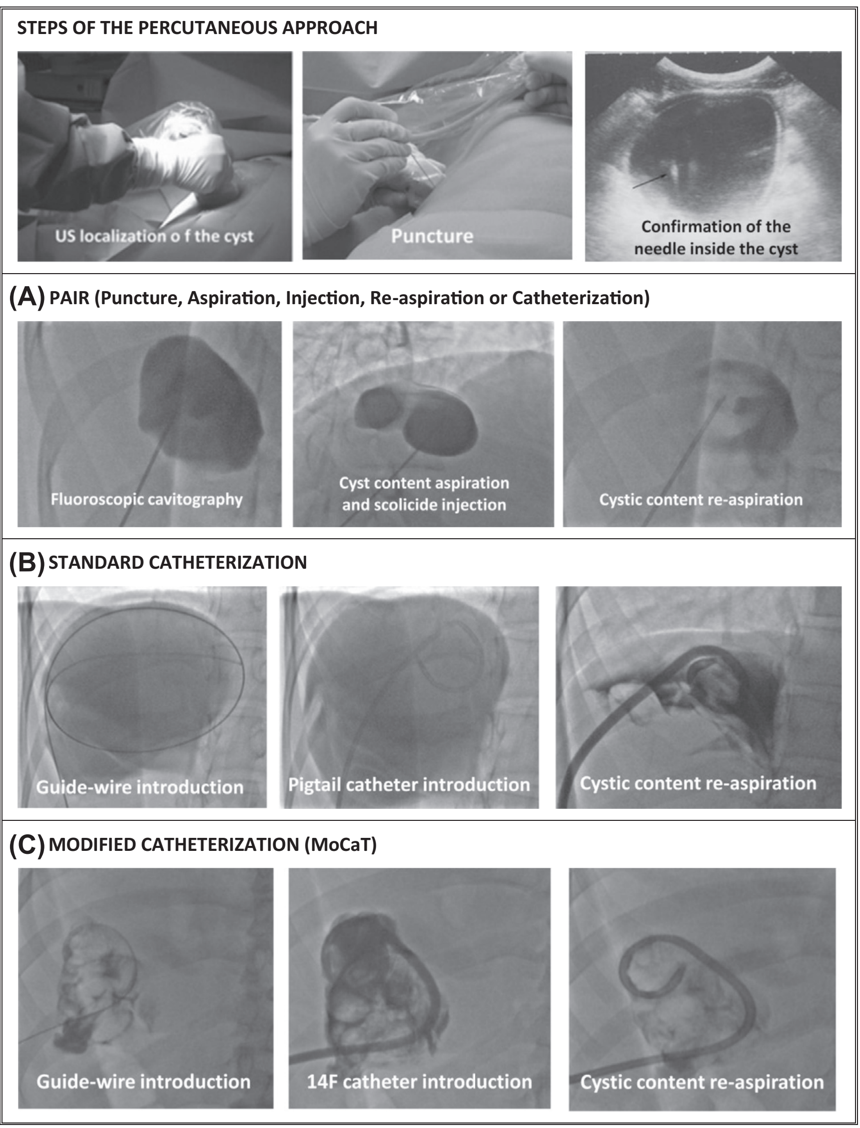


Figure 31 Percutaneous techniques to treat hepatic cystic echinococcosis.

be used to treat cysts of any diameter. However, it is the preferential treatment for cysts larger than 10 cm in diameter and/or with a liquid content greater than 1000 mL. The introduction of a catheter allows draining of cyst fluid, with the catheder removed at the conclusion of the procedure.

Modified Catheterization Technique (Akhan et al., 2007b): This procedure results in removal of the parasitic membranes in addition to the cyst's content.

The three above techniques are schematically illustrated in Fig. 31, and their indications, benefits and risks/disadvantages are shown in Table 2. The procedures are described in detail in Annex 1.

Success of percutaneous treatment is defined, by imaging, as a reduction in the cyst's size and volume, thickening of the cyst's wall and solidification of the remaining structure (Akhan et al., 1993, 1996; Akhan and Ozmen, 1999). Furthermore, the procedure is considered to be successful if the cyst is no longer viable and complications, such as abscess formation, have not occurred.

1.3.2.3 Surgical approaches

The objective of surgery in the treatment of CE is the removal of the cyst (Sayek and Onat, 2001; Buttenschoen and Carli Buttenschoen, 2003; Yagcı et al., 2005). Cystectomy ideally should be total, to diminish relapses and complications. It can be performed through laparotomy or laparoscopy, both by open or closed methods. In both options the dissection is made on the outside of the adventitia ('pericyst'), to guarantee a complete cyst removal.

Total cystectomy: This technique entails the complete excision of the cyst, with the goal of avoiding relapse. The CE cyst can be opened in situ, referred to as 'open method'. If the cyst remains closed during operation the procedure is called 'closed method' (Menezes da Silva, personal communication). The open method is done by opening the cyst followed by aspiration and the removal of its content (Fig. 32). The 'closed method' consists of the complete removal of the cyst, i.e., the parasite and the host tissue that surrounds it ('pericyst').Peng et al. have recently developed the 'periadventitial technique'. The dissection is made in the virtual space between the adventitia and the sane hepatic tissue. The authors claim that this technique has fewer complications, while being as effective to remove all parasitic tissue (Lv et al., 2015). If a total cystectomy is not achievable due to potential damage to vascular structures, a partial cystectomy may still be used to achieve parasite removal.

Partial cystectomy: This is a nonradical procedure and results in the presence of a residual cavity that can originate infection and abscesses. Simple drainage with suction and filling with epiploon (omentoplasty) are options to reduce the risk of complications.

Table 2 Percutaneous and surgical procedures to treat uncomplicated cystic echinococcosis (CE) of the liver

Method/ technique	Structure targeted	Indication WHO–IWGE stage	Type of intervention/ benefits	Risks, disadvantages	Medical requirements	References
Percutaneous procedures						
PAIR[a]	Chemical sterilization of parasitic layers; aspiration of cystic fluid	CE1 CE3a	Percutaneous technique Least invasive, less painful, early discharge	Minimal complications	Radiology-guided interventional team	Ben Amor et al. (1986) and Filice et al. (1990)
Standard catheterization	Chemical sterilization of parasitic layers, aspiration of most of the cyst's fluid contents	CE1 CE3a	Percutaneous technique; management of large-size cysts and assessment and treatment of cysto-biliary communications than PAIR	Longer hospital stays; More risks of infection than PAIR	Radiology-guided interventional team	Khuroo et al., 1991, Akhan et al. (1993, 1996), and Ustünsöz et al. (1999)
Modified catheterization (MoCaT)[b]	Sterilization, aspiration, irrigation and removal of parasite and solid components (parasitic layers and daughter cysts if any)	CE2 CE3b	Percutaneous technique, possibly combined with endoscopy; Minimal invasive; avoidance of surgery	Relatively high infection/abscess rates Long term outcome unknown	Radiology-guided interventional team, and interventional/ endoscopy team to perform papillotomy	Akhan et al. (2007a) and Schipper et al. (2002)
Surgical approaches						
Partial cystectomy	Removal of parasite-derived cyst components and part of adventitial tissue	CE2 CE3b In case of contraindication for catheterization	Laparotomy or laparoscopy; Nonradical procedure	Small remnants of parasitic layers often left over; High relapse rate	General surgery team	Manterola et al. (2002) and Atmatzidis et al. (2005)

Total cystectomy • Open method • Napalkoff method • Peng method	Removal of entire cyst	Middle- and large-sized CE1 CE2 CE3b	Laparotomy or minimally invasive surgery; Radical procedure	General peri-and postoperative risks; Bleeding; Hospitalization	Experienced liver surgery team	Atmatzidis et al. (2005) and Lv et al. (2015)
Hepatic segment or lobectomy	Removal of entire cyst and part of the sane liver	Giant cysts	Laparotomy; Radical procedure	General peri-and postoperative risks, i.e., bleeding and general complications of major surgery; Hospitalization	Specialized liver surgery team Not available in all hospital settings	Sayek and Onat (2001)

[a] Puncture, Aspiration, Injection, Reaspiration.
[b] Modified Catheterization Technique.

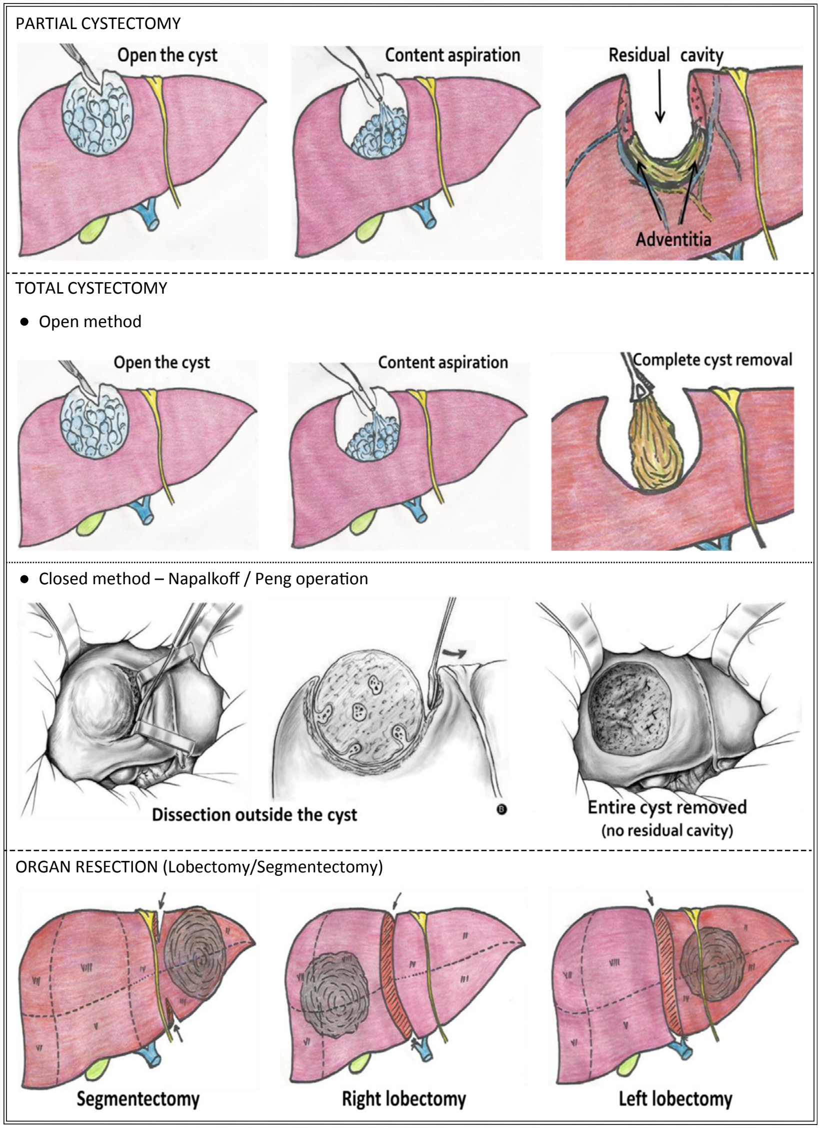


Figure 32 Principal surgical approaches to treat hepatic cystic echinococcosis.

Hepatic resection (segment- or lobectomy): This surgical method is the most radical treatment option for CE and may be indicated for giant cysts with a high risk of ischaemia of the remaining hepatic tissue. Although the morbidity and mortality associated with CE surgery have diminished, they

cannot be overlooked (Aydin et al., 2008). To prevent relapses, it is very important to protect the surgical field with pads soaked with scolecidal solutions that are nontoxic to the biliary tree (Aydin et al., 2008; Ezer et al., 2008; Akbulut et al., 2010; Prousalidis et al., 2012). Both radical methods have higher risks, perioperatively, but fewer rates of complications and relapses. On the other side, nonradical methods have fewer risks, intraoperatively, but a higher rate of complications and/or relapses. The main advantage of radical procedures is the definite cure of CE.

1.3.2.4 Watch-and-wait approach

Long-term follow-up of patients with US imaging has increased clinicians' confidence that, in selected cases, CE treatment can be put on hold. This is an option for uncomplicated inactive cysts, including most CE4 and all CE5 cysts. These cyst types appear to either remain stable in size or degenerate over time and do not compromise organ functions or cause patient discomfort. However, these should undergo long-term US follow-up for at least 10 years (Junghanss et al., 2008). The watch-and-wait approach may also be suitable for other cyst types (e.g., small CE1 cysts). However, additional prospective studies are needed (Brunetti et al., 2011).

1.3.2.5 Treatment of complicated hepatic cystic echinococcosis

In patients with complicated cysts (e.g., rupture, development of a cyst-biliary fistula, compression of vital organs and vessels, haemorrhage, bacterial superinfection, giant cysts) surgery is the treatment of choice. Intrabiliary rupture of LCs is a frequent complication of surgically managed hepatic CE cases (5–25%) (El Malki et al., 2010). Cyst rupture occurs into the right biliary duct 55–60% of the time and into the left duct 25–30% of the time. Cyst may also rupture into the gallbladder. Biliary fistulae can be occult (10–37%) or frank (3–17%), resulting in biliary obstruction (Atli et al., 2001). Asymptomatic patients with larger cysts are at a higher risk for cyst communication with the biliary tree. US and CT have diagnostic sensitivities, for the detection of biliary complications, of 78.4% and 85.7%, respectively (Akhan, personal communication). MRCP has become an effective, noninvasive and useful diagnostic tool in difficult cases. Endoscopic retrograde cholangiopancreaticography (ERCP) is currently considered the gold standard for confirmation of biliary complications.

While surgical intervention of biliary fistulae is still common, percutaneous and endoscopic approaches have shown promise in several publications of patient series (Chautems et al., 2005; Vaz et al., 2012; Zeybek et al., 2013). An occult fistula can be treated by percutaneous drainage alone. This procedure can be associated with endoscopic biliary clearance (papillotomy). Frank biliary fistulae can be treated endoscopically, followed by percutaneous drainage, if deemed necessary. Cyst-biliary communication can be detected prior to surgery. If the communication is detected intraoperatively, it should be precisely localized and the biliary tree explored using dye or radiopaque markers. The communication can be treated by suturing and/or drainage of the cystic cavity or by drainage of the common bile duct. Biliary-intestinal anastomosis and/or partial liver resection are sometimes necessary. Sphincterotomy alone is not an adequate treatment since spontaneous closure of fistulae from cysts with calcified walls is rare.

1.3.3 Treatment of thoracic cysts

Surgery: Surgical interventions for thoracic cysts should be as conservative as possible. However, radical procedures are required for extended parenchymal involvement, severe pulmonary suppuration and other complications. Surgical approach varies with cyst localization (Isitmangil et al., 2002). Video-assisted thoracoscopic removal of cysts has been utilized in children and in cases with small cysts located along the periphery of the right lower lung lobe (Chowbey et al., 2003; Mallick et al., 2005). Pleural cavity drainage may also be performed during this procedure. During surgical treatment for pulmonary CE the cyst is punctured, the parasitic contents are aspirated and a scolecide is introduced for 10–15 min prior to cyst removal. In the case of an active perifocal process, the resection of lung tissue is required. In patients with lung cysts accompanied by cysts on the diaphragmatic surface of the liver cyst a phrenotomy should be performed to allow cyst removal. If both the right and the left lung lobes contain cysts, surgery is performed on one side per session, with 3–6 months between the procedures.

Benzimidazoles treatment: Drug treatment is used in pulmonary CE patients who are considered poor surgical candidates, including those with unresectable and multiple cysts. BMZ used alone has shown a good efficacy on small, uncomplicated lung cysts (Anadol et al., 2001; Dogru et al., 2005). However, a BMZ should be avoided preoperatively for larger lung cysts, as antiinfective therapy may promote rupture due to degenerative changes in the cyst wall. BMZ-associated cyst rupture typically occurs within 10 days

after the start of treatment but may be observed 1–2 months later after beginning treatment (Todorov et al., 2005).

1.3.4 Treatment of cystic echinococcosis cysts in other organs

CE cysts located in other organs other than the liver or lungs are rare and management, of these cysts, continues to be a challenge. Small case series often provide guidance for possible treatment options.

Spleen: Rupture of splenic cysts into the peritoneal cavity is common. Historically, surgery was the only accepted treatment modality for splenic CE, with several surgical techniques ranging from splenectomy to spleen-sparing surgical resection. Percutaneous treatment of splenic CE has also gained acceptance in the last decade when used in conjunction with a scolecidal agent (Akhan et al., 2016a,b).

Peritoneal cavity: Presence of CE cysts in the peritoneal cavity is usually related to the spillage of a ruptured cyst's content, either spontaneously or during surgery. If the cysts are very large or are located in or near vital organs, the treatment should combine BMZ application and surgery. Prolonged treatment with BMZ may be the only option in many of these cases, with the goal of reducing the number and/or size of the cysts. Percutaneous treatment has shown promise for selected cases, although cyst content spillage accompanied by anaphylaxis is a risk with this procedure (Akhan, personal communication).

Kidney: Surgery has been the historical treatment of choice for renal CE cysts. The type of surgery chosen for the management of renal echinococcosis depends on the individual patient. Nephrectomy and partial nephrectomy are most common utilized, with marsupialization also appropriate for some cases. Cystectomy is possible in 75% of the renal cases. Nephrectomy should be reserved for cases in which a kidney is not functional due to a cyst rupturing into the renal pelvis or when there are complications due to secondary bacterial infection. Renal cysts have also been successfully treated by percutaneous techniques for the last 20 years (Öner et al., 1995; Akhan et al., 1998a).

Bone and spine: Bone CE is less sensitive to BMZ than cysts in other locations, and long-term administration of BMZ dosages may be necessary (Neumayr et al., 2013b). The most effective treatment is radical resection of the affected bone. Multiple recurrences, with the need for repeated surgical procedures, are common. Patients with serious complications, such as spinal involvement, fistulae, and acute and chronic osteomyelitis, have a poor prognosis.

Brain: For CE of the brain the most appropriate treatment method is complete surgical removal of the cyst without rupture. However, if this is not possible the cyst should be removed after puncture and aspiration of the cyst's contents. Removal of large cysts may be complicated by intraoperative rupture, often leading to death or subdural haemorrhage with long-term sequelae (Duisbanbai et al., 2010).

Intravascular sites: Surgery is the treatment of choice for intravascular CE. Venous filters are used to prevent cyst dissemination. If complete removal of a cyst is possible the prognosis is good, with a low rate of recurrence (Diaz-Menendez et al., 2012).

1.3.5 Follow-up management

All treatment plans for CE should include long-term patient follow-up to detect changes in the cyst and possible relapses. Four follow-up visits, including imaging examination, should be conducted during the first year posttreatment. If the patients's condition appears to be stable, this number can decrease to two visits during the second year posttreatment and then to once a year for at least 10 years (Brunetti et al., 2010).

1.3.5.1 Imaging

Various imaging modalities, such as US, CT or MRI, may be used to follow CE cases. The choice of imaging method is based on cyst location, with US the tool of choice for hepatic CE, CT the tool of choice for pulmonary CE, and MRI the tool of choice for brain CE. During imaging evaluation the size, volume, content and wall of the cyst should be assessed (Akhan et al., 1993). After the cyst is punctured, detachment of the parasite's membrane and solidification of the cyst can be evaluated by US. This solid remnant (pseudotumor) is shown in Fig. 33. A hyperechoic double contour line surrounded by a hypoechoic rim is seen on US in some cases treated with ABZ (Akhan et al., 1996; Ustünsöz et al., 1999; Men et al., 1999; Azeemuddin et al., 2005; Marrone et al., 2012; Nunnari et al., 2012). Treatment with ABZ also results in degenerative changes, such as reduction in the fluid volume of the cyst, parasite-layer detachment, development of a pseudotumoral appearance, calcifications (in 1–3 years) and rarely complete disappearance of the cyst (Franchi et al., 1999). After surgery, attention should be paid to the postoperative cavity and possible complications related to cyst communications with the biliary tree, which could have been missed at the time of surgery. In all cases, patients should be monitored for possible recurrence of the cyst in the same organ and in neighbouring organs.

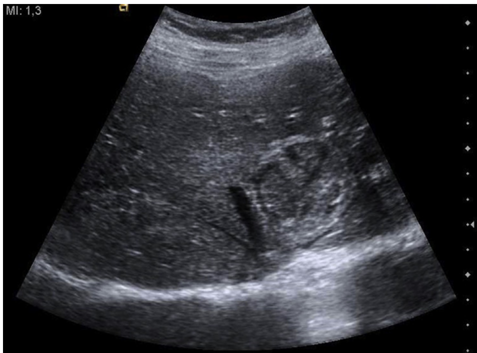


Figure 33 Abdominal ultrasonography. Hepatic cystic echinococcosis lesion located in segment 4A. The remnant has a pseudosolid appearance with mixed echogenicity.

Patients who undergo watch-and-wait approach for hepatic CE should be examined once a year by US (Brunetti et al., 2010).

1.3.5.2 Serology

Serology can be used, in conjunction with imaging, to monitor for cyst recurrence (Tamarozzi et al., 2014a). A slow decrease in specific antibody levels is usually observed after radical surgery (Ben Nouir et al., 2008). In contrast, after most of other therapeutic procedures, antibody levels do not typically decrease appreciably (Rigano et al., 2002). Additional information regarding serological testing for CE can be found in Chapter "Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals" by Siles-Lucas et al., 2017.

1.3.5.3 Failure in cystic echinococcosis management

Anaphylactic reactions: Severe allergic reactions can include urticaria/oedema, respiratory symptoms and anaphylactic shock. In some cases, CE patients may initially present with these manifestations (De Wispelaer et al., 2011; Fabian et al., 2015). Minor spillage of cyst contents can be sufficient to cause anaphylaxis. However, in most cases anaphylaxis is caused by a rupture of a cyst during surgical or percutaneous intervention. Fortunately, as percutaneous techniques have improved, anaphylaxis secondary to percutaneous management is now considered rare (Neumayr et al., 2011). That said, when performing surgery or cyst puncture, clinicians should be aware of the risk of anaphylaxis and prepared to address this possible complication.

Cysto-biliary leakage: Postoperative bile leaks can occur after surgical treatment of LCs. This complication is most common after nonradical surgery

(Zeybek et al., 2013), resulting in significant morbidity, including biliocutaneous fistulae, bilomas and bile peritonitis, which occur in 4–28% of hepatic CE cases treated surgically (Kapoor and Nundy, 2012). Ideally an intraoperative cholangiogram should be performed to identify cyst-biliary communications. If leakage is detected, ligation should be performed along with biliary decompression using a T-tube, if necessary. A majority of fistulae resolve spontaneously, but sometimes biliary drainage (endoscopic or surgical) is required.

Therapeutic failure: Therapeutic failure can be the result of recurrence or infection. In the case of recurrence a second intervention should be pursued. If infection of the surgical cavity occurs, treatment with percutaneous drainage and appropriate antibiotics is indicated.

1.3.6 Confounding conditions

It is unclear how immunosuppression (e.g., coinfection with HIV/AIDS) impacts CE occurrence or progression (Capdevielle, 1984; Gruener et al., 2008; Wahlers et al., 2013; Ran et al., 2015). While CE cysts in unusual locations have been reported in immunocompromised patients (Erayman et al., 2011), there is no evidence that these patients have an increased risk of CE in unusual sites. Unexpectedly, in small retrospective case series in Western China, an increase in the CD4 T-lymphocyte count was observed after the surgical removal of the CE cysts in patients with AIDS (Ran et al., 2015). The observation of peritoneal CE in a cat infected with feline immunodeficiency virus (FIV) (Armua-Fernandez et al., 2014) suggests that immune suppression may actually facilitate the development of the metacestode as seen in this unlikely intermediate hosts. Coinfection with CE and aspergillosis has also been reported in immunocompromised patients, especially those with pulmonary CE (Kosmidis and Denning, 2015; Koçer et al., 2008; Vasquez et al., 2008, Deshmukh et al., 2009; Fifer et al., 2012; Agarwal et al., 2013). This could be due to the anatomical disturbances caused by the cyst and/or by local immune tolerance due to the presence of the metacestode. There are only a few studies looking at the association of CE with cancer (Esendagli and Abbasoglu, 2015; Tez and Tez, 2015). Most studies have focused on the diagnostic and therapeutic challenges associated with hepatocellular carcinoma (Li et al., 2015; Göya et al., 2014; Bakoyiannis et al., 2013). At present, there appears to be more evidence regarding an association with cancer and AE than between cancer and CE (Chauchet et al., 2014). However, studies are currently

underway to evaluate if CE predisposes certain populations to developing cancer (Oikonomopoulou et al., 2014; Turhan et al., 2015).

1.4 Outcome and prognosis

If performed appropriately, percutaneous aspiration of hepatic CE is now recognized as a safe treatment for certain cyst types (Neumayr et al., 2011). Specific outcome should be evaluated by WHO-IWGE cyst stage (see below).

1.4.1 Outcomes for hepatic CE1 and CE3a cysts after percutaneous procedures

Several small case series, focussing on the short-term follow-up of CE1 and CE3a cysts undergoing the PAIR technique, have been published (Ben Amor et al., 1986; Bret et al., 1988; Filice et al., 1990; Gargouri et al., 1990; Khuroo et al., 1993; Acunas et al., 1992; Giorgio et al., 1992; Bastid et al., 1994). The conclusion, from these case series, has been that PAIR is a safe and effective treatment option for CE1 and CE3a cysts. In a prospective study, 33 patients with hepatic CE were randomly assigned to receive percutaneous drainage, ABZ plus percutaneous drainage or ABZ alone. The study concluded that percutaneous drainage with ABZ therapy was most effective method assessed for the management of hepatic cysts (Khuroo et al., 1993).

The first paper evaluating the long-term results of percutaneous treatment (mean follow-up of approximately 32.5 months) was published by Akhan et al. (1996). This study found that 10% of individuals who underwent percutaneous treatment had serious complications. In addition, study patients stayed in the hospital for an average of 3 days and 2% had a recurrence of their disease within the study timeframe. Üstünsöz et al. (1999) treated 106 hepatic CE cysts in 72 patients ('Gharbi' type I-II-III) using PAIR or catheterization techniques with good results. The authors noted that cysts of two PAIR patients had recurred at 3-months and 6-months follow-up examinations. In a similar study, published that same year, all patients were successfully treated with a percutaneous method, with the exception of one patient who died due to anaphylactic shock (Men et al., 1999).

There is one prospective randomized trial that compared the results of the PAIR technique with traditional surgery (Khuroo et al., 1997). The authors concluded that PAIR was associated with fewer complications (32% with PAIR and 84% with surgery) and a shorter hospital stay (4.2 ± 1.5 days with PAIR and 12.7 ± 6.5 days with surgery). Thus PAIR combined with

ABZ has been found to be an effective and reliable treatment method for certain stages in noncomplicated hepatic CE. A meta-analysis comparing the results of 769 patients treated with PAIR and 952 patients treated surgically, concluded that PAIR was associated with lower frequency of major complications (7.9% with PAIR and 25.1% with surgery), lower frequency of recurrence (1.6% with PAIR and 6.3% with surgery) and shorter hospital stay (2.4 days with PAIR and 15 days with surgery (Smego et al., 2003). Finally, Brunetti et al. (2004) published a review of the literature on 20 years of percutaneous treatment for CE. Their conclusion was that percutaneous treatment of CE is a safe therapeutic alternative to the traditional surgical removal of CE1 and CE3a cysts. This conclusion has been corroborated in more recent reviews (Junghanss et al., 2008; Brunetti et al., 2010; Gupta et al., 2011). Prospective studies are still needed to compare the various surgical and percutaneous approaches and to determine the optimal schedule for ABZ administration.

Studies evaluating the long-term effectiveness of ABZ on CE1 and CE3a cysts show treatment successes ranging from 50% and 75%. However, differences in inclusion criteria, patient compliance, reevaluating interval and how success was defined make comparing results difficult (Salinas et al., 2011). Cyst size may also impact the success of treatment with ABZ, with some studies suggesting that cysts smaller than 6 cm in diameter are most successfully treated with ABZ alone (Stojkovic et al., 2009). In a study by Franchi et al. (1999) 929 hepatic CE cysts were treated with a BZM. The authors found that the most frequent US findings were a reduction in the liquid component, and the appearance of a pseudosolid mass. A volumetric reduction in the cyst and detachment of parasite membranes occurred more frequently in CE1 cysts as compared to CE3a cysts.

1.4.2 Outcomes for CE 2 and CE 3b cysts after percutaneous Procedures

There are several studies evaluating the outcome of CE2 and CE3b cysts after treatment with PAIR or a catheterization technique (Akhan et al., 1996; Kabaalioglu et al., 2006; Giorgio et al., 2001; Filice et al., 1990; Men et al., 1999; Akhan et al., 1998a). In one study, recurrence occurred in 61.5% of patients during follow-up after the PAIR technique (Kabaalioglu et al., 2006). In another study, 58 multivesicular cysts (CE2 or CE3b) in 30 patients underwent double-percutaneous-aspiration-and-ethanol-injection (D-PAI). Local recurrences were observed 14 patients with 19 multivesicular cysts. Recurrence rate was calculated for cysts with 32.2% of the cysts and

for patients with 46.6% in this study (Giorgio et al., 2009). Modified percutaneous techniques, for the treatment of CE2 and CE3b, have been used since the early 1990s (Saremi, 1992; Saremi and McNmara, 1995; Wang et al., 1994). While short-term results of percutaneous puncture, drainage and curettage (PPDC) technique were promising (Wang et al., 1994; Vuitton et al., 2002), long-term follow-up (over 10 years) showed a significant number of recurrences (Wang and Wang, personal communication).

At the beginning of the 2000s the percutaneous evacuation (PEVAC) gained popularity. With this method, all solid cyst components are evacuated using a thick suction catheter (Schipper et al., 2002). However, anecdotally, there was a high frequency of cysto-biliary fistulae with this method. A small case series of eight Lebanese patients with complex Gharbi type IV hepatic cysts – most likely including CE3b cysts – reported no major complications with the PEVAC method. However, patients were only followed for 1–48 months (Haddad et al., 2000).

The MoCaT technique was first described for the treatment of CE2 and CE3b by Akhan and Koroglu (2007). The outcomes of 26 CE2 and CE3b patients treated with MoCaT were retrospectively compared with the outcomes of patients treated with PAIR (n = 26) or a catheterization technique (n = 23), with a mean follow-up of 80.1 months. The authors concluded that the MoCaT technique resulted in lower recurrence rate compared to the other percutaneous techniques. However, MoCaT also had a higher likelihood of complications compared to the other evaluated techniques. Prospective studies are needed to better evaluate the effectiveness of the currently available treatment modalities for hepatic CE2 and CE3b cysts (Akhan et al., 2016a,b).

1.4.3 Outcomes for CE 4 and CE 5 cysts

CE4 and CE5 are, in principle, not indications for percutaneous treatment. Spontaneous degeneration of these cysts is the rule, and the watch-and-wait approach has been recommended for such cysts (Brunetti et al., 2010).

1.4.4 Outcome for nonhepatic CE cysts

Surgery is the most commonly recommended treatment option for patients with one or more giant pulmonary CE cysts. If there are multiple small (<5 cm in diameter) CE cysts in the lung, treatment with a BMZ should be in the first line of treatment (Akhan, personal communication).

Percutaneous treatments have been successfully performed for CE cysts located in the lung (Akhan et al., 1994b), kidney (Akhan et al., 1998a), peritoneal cavity (Akhan et al., 2016a,b), spleen (Akhan et al., 2007b), orbital cavity (Akhan et al., 1998b), soft-tissues (Akhan et al., 2007b), adrenal glands (Akhan et al., 2011) and parotid glands (Akhan et al., 2002). For all cyst locations, treatment options should be based on local expertise and individual patient parameters.

1.5 Burden of cystic echinococcosis

1.5.1 Nonmonetary burden: evaluation of the disability adjusted health years losses associated with cystic echinococcosis

Estimation of the socioeconomic impact of CE on a population is an important step in understanding both the public health and economic impacts of this zoonotic disease on a defined region. Impact can be presented in both nonmonetary and monetary terms. This information helps to show the need for control interventions as well as provides a means to assess the success of these interventions.

In an effort to compare different diseases and conditions across populations, measures have been developed to estimate the nonmonetary burden of human diseases (Carabin et al., 2005; Budke et al., 2011). One of the most utilized metrics is the disability adjusted life year (DALY), which was designed to be an objective, population-based measure. DALYs assess both the disability and early mortality associated with the condition of interest and are obtained by summing years of life lost from premature death and healthy years lost due to disability. The DALY is a negative concept, with one DALY being the equivalent of one year lived completely disabled (analogous to death) (Gold et al., 2002). Therefore, control strategies would aim to minimize DALYs lost. DALYs were first developed for the Global Burden of Disease (GBD) Study to evaluate the nonmonetary burden of a variety of infectious and noninfectious conditions, as well as risk factors, on predefined regions of the world (Murray et al., 1994).

The first global estimate of the nonmonetary burden of CE was conducted in 2006 and used the DALY to evaluate losses associated with human cases (Budke et al., 2006). Without adjusting for underreporting an estimated 285,400 DALYs were lost due to CE. However, after the value was adjusted for underreporting, the estimate increased to more than 1 million DALYs lost, which is similar to evaluations for diseases such as Chagas disease, dengue and onchocerciasis (Budke et al., 2006). The 2010 and 2013 GBD Studies included DALY estimates for echinococcosis

and CE, respectively (Murray et al., 2012, 2015). However, methodological decisions make interpretation of the 2010 GBD values problematic in that the study attempted to combine CE and AE into a single estimate (Murray et al., 2012). Due to the different life cycles of *Echinococcus granulosus* and *Echinococcus multilocularis*, as well as differences in clinical course, there were substantial issues in extrapolating estimates to data-poor regions and assigning appropriate disabilities to affected populations. The 2013 GBD Study focused solely on CE, thereby, eliminating many of these issues (Murray et al., 2015). However, data gaps remain a problem. In parallel to the 2010 GBD Study the World Health Organization's Foodborne Disease Burden Epidemiology Reference Group (WHO-FERG) published global CE burden estimates for the year 2010 (Torgerson et al., 2015).

Relatively few investigator-driven studies have been conducted to estimate the nonmonetary burden of CE in specific regions. The first study using the DALY to evaluate the burden of CE was conducted in a remote and highly endemic region of the Tibetan Plateau of Western China (Budke et al., 2004). This study helped to convey the magnitude of this chronic disease on populations who could not readily obtain medical treatment. Other studies have since been conducted in diverse geographic locations, including Peru, Sardinia, Nepal, and Xinjiang, China (Moro et al., 2011; Mastrandrea et al., 2012, commented on Tamarozzi et al., 2015a; Devleesschauwer et al., 2014; Wang et al., 2012).

1.5.2 Monetary burden; human health cost and livestock-associated economic losses due to cystic echinococcosis

In addition to metrics such as the DALY the impacts of diseases are also often expressed in terms of costs to individuals and society. Estimates of the burden of zoonotic diseases, such as CE, that affect both human and livestock populations should include cost estimates for losses in all impacted species. Human health costs are typically divided into direct and indirect costs. *Direct costs* are associated with the diagnosis and treatment of patients. Diagnostic testing, including advanced imaging and serology, as well as the cost of medications, medical consultations, surgery and hospitalization would be considered direct costs. In contrast, *indirect costs* include costs associated with over-the-counter medications, traditional medicine and transportation to and from medical treatment as well as wage and productivity losses attributable to an inability to work due to clinical manifestations or visits to clinics and hospitals. Indirect costs are often also extended to the costs associated with family members taking care of the patient.

CE-associated treatment costs can be assessed using Diagnosis-Related Group reimbursements to hospitals or by calculating the cost per patient from a representative sample of CE patient medical records over a defined time period. This second method has been used in studies conducted in Uruguay, Jordan, the United Kingdom and Argentina (Torgerson et al., 2000, 2001; Torgerson and Dowling, 2001; Bingham et al., 2016). Human CE-associated indirect costs are often calculated using either wage data or per capita GDP, with wage data providing a more accurate estimate of the impact of CE on infected individuals and their families.

Livestock-associated economic losses include direct costs, resulting from the condemnation of cyst-containing offal, as well as indirect costs due to production losses, including decreases in carcass weight, milk production, fibre production and fecundity (Torgerson, 2003). Direct costs can be calculated using local market values for the condemned organs (Torgerson et al., 2001; Benner et al., 2010; Harandi et al., 2012). In contrast, livestock production losses can be difficult to estimate due to the limited number of controlled studies that have evaluated these types of losses (Torgerson, 2003).

An advantage of using a monetary approach is that it allows for the incorporation of both human and livestock-associated losses into the same estimate. In lower income countries, it is particularly important for burden of disease estimates to include livestock-associated economic losses since livestock production represents a substantial proportion of the income for small scale agriculturalists and pastoralists (Carabin et al., 2005). In addition, including estimates for both human and livestock-associated losses can lead the way to cost-sharing between the agricultural and public health sectors (Roth et al., 2003). A disadvantage of using only a monetary approach is that it may underestimate the impact of the disease in very poor regions, where unadjusted income and livestock values are substantially less than in wealthier communities (Budke et al., 2006).

The monetary burden of CE has been estimated globally as well as at the country and regional levels. Based on a 2006 estimate of the global burden of CE, monetary annual losses attributable to human CE were estimated to be $193 million and increased to $764 million when adjusted for underreporting (Budke et al., 2006). In the same study, livestock-associated monetary annual losses were estimated to be $142 million when only direct costs attributable to liver condemnation were included (Budke et al., 2006). However, this amount increased to approximately $2.2 billion when

productivity losses were included and the estimate was adjusted to account for underreporting.

Although CE-associated monetary losses have been estimated for several countries, the lack of a standardized methodology makes comparisons difficult. As a result the categories of costs included may vary greatly between studies. While a few studies have estimated only livestock-associated costs, most studies with a livestock component also estimate monetary losses associated with human CE (Ahmadi and Meshkehkar, 2011; Sariozkan and Yalcin, 2009; Torgerson et al., 2000, 2001; Budke et al., 2005; Majorowski et al., 2005; Benner et al., 2010; Moro et al., 2011; Harandi et al., 2012; Venegas et al., 2014). These studies have all shown that CE can have a substantial impact on both the human and livestock sectors in endemic regions (Craig et al., 2007).

1.5.3 Main gaps in the assessment of monetary and nonmonetary burden of cystic echinococcosis

Substantial data gaps continue to exist when assessing the monetary and nonmonetary burden of CE. One of the most glaring gaps is a lack of information on the frequency of infection in both developing and developed countries. While there are countries that claim to collect and make data available on the number of new human cases identified and treated each year, these values are frequently underreported due to the lack of a centralized data collection system (Tamarozzi et al., 2015). In addition to the difficulty in collecting and conveying data on treated cases, many developing countries do not have the medical infrastructure in place to diagnose patients in rural locations. Therefore, even though global burden estimates currently exist, burden estimation extrapolation to data-poor regions continues to be problematic.

In addition to lack of frequency data, there are also issues related to quantifying the level disability attributable to the full range of clinical manifestations related to human CE. To date, most studies have focused on the disability associated with hepatic and pulmonary cases of CE without taking into account disability associated with cysts in other parts of the body, such as the brain. On the livestock side, while many official abattoirs collect information on cysts detected on postmortem examination, in many parts of the world animals are frequently slaughtered without the benefit of inspection. There is also a true dearth of information on how CE affects overall livestock production and product quality.

2. ALVEOLAR ECHINOCOCCOSIS

2.1 Introduction

Human AE is a zoonosis caused by the metacestode stage of the so-called dangerous fox tapeworm *E. multilocularis*. This parasite is predominantly perpetuated in a wildlife-cycle, with carnivores as definite hosts and small mammals as intermediate hosts. The geographic distribution is restricted to the Northern Hemisphere, with Central Europe, North America and Japan traditionally recognized as endemic regions (Eckert et al., 2011). However, focus areas for AE research and control have changed over the past several decades as highly endemic areas in Western China and adjacent regions have been identified (Vuitton et al., 2003). Furthermore, expansion of the distribution area of *E. multilocularis* into Northern, Eastern and Western Europe has been reported, with the disease in humans occurring in countries previously regarded as AE free (Davidson et al., 2012).

In 2010 the global burden of AE was estimated to be approximately 18,200 new cases/year (Torgerson et al., 2010), with 91% of these cases occurring in rural communities on the Qinghai-Tibet Plateau of Western China (Wang et al., 2014). As a result of the public health threat caused by this disease the WHO has listed AE as a neglected zoonotic disease (NZD) (http://www.who.int/echinococcosis/en/).

Humans can accidently acquire the infection through ingestion of eggs shed in the feces of a definite host. Infected individuals typically develop a silently-progressing hepatic disease that clinically behaves like a tumour with infiltrative growth and the potential for metastasis (Kern, 2010). Thus AE is often acknowledged as one of the world's most lethal chronic parasitosis due to the high fatality rates in untreated patients (Ammann and Eckert, 1995). As AE is an NZD, clinical diagnosis and management remain a challenge. The metacestode that causes AE has a different biological behaviour as compared to the larva of *E. granulosus* s.l., the causative agent of CE. The 'malignant' growth of the larva leads to infection of the liver, infiltration of neighbouring organs including lymph nodes (Buttenschoen et al., 2009a) and the formation of distant metastases. The lesions are often solid, with or without necrotic centre, and may have cystoid/microcystic components. The lesions always consist of small cysts which may not be visible in images. To better describe the anatomical extension of the disease, the WHO-IWGE developed the PNM classification, where P1-4 indicates the location of the Parasite in the liver;

N indicates if Neighbouring organs are involved and M indicates if Metastases are formed (Kern et al., 2006). The PNM classification aims to guide clinicians along a decision tree, and thus, to optimize the management of the disease.

Various imaging tools can be utilized to help make a diagnosis of AE, with serological testing playing a supportive diagnostic role. Imaging studies, including ^{18}F-FDG-PET/CT, should be used to guide the management of this chronic disease. Invasive measures are often performed for the differential diagnosis of hepatic malignancies. Diagnosis confirmation of AE is based on pathological or immune-histological findings (Barth et al., 2012) and molecular tools (see Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017). However, misdiagnosis of AE is common and often results in mismanagement of the patient and life-long sequelae. One of the major obstacles to appropriate patient management is the inability of many physicians and surgeons to differentiate AE from CE. This holds true in endemic and nonendemic regions.

AE can be cured by radical surgery if detected at an early stage. In European cohorts the rate of resectability was reported to range from 20% to 50% (Bresson-Hadni et al., 2000; Kadry et al., 2005; Schweiger et al., 2007; Buttenschoen et al., 2009c; Grüner et al., 2017). Noncurative resections are discouraged and nonsurgical interventions should be pursued in patients with lesions that cannot be completely resected (Buttenschoen et al., 2009b; Bresson-Hadni et al., 2006; Frei et al., 2014; Graeter et al., 2015; Ambregna et al., 2017). Liver transplantation remains a rescue measure in selected cases (Bresson-Hadni et al., 2011). BMZs are the backbone of medical treatment. New drugs are being identified, but clinical application has rarely been pursued (Hemphill et al., 2014; Vuitton and Bresson-Hadni, 2014). In general, and especially in remote locations where incidence is high and access to health-care facilities is difficult, AE patients may present with advanced disease and require life-long medical treatment and follow-up. Current treatments have substantially improved the prognosis of AE for patients living in countries with well-developed health-care systems (Torgerson et al., 2008). However, even in European countries, more human AE cases are anticipated during the next decades (Gottstein et al., 2015). The increase in number of cases and disease burden might, in part, be attributable to number of immunosuppressed individuals. A relationship between immunosuppression and AE infection has recently been established (Chauchet et al., 2014). In most of the endemic areas, AE still remains a lethal disease (Ayifuhan et al., 2012).

2.2 Clinical diagnosis and definitions

2.2.1 Metacestodes in the human host

2.2.1.1 Growth, structure and Size

After accidental ingestion, embryos (oncospheres) hatch from the parasite eggs in the upper gastrointestinal tract, penetrate the gut wall, travel via blood or lymph and are trapped mainly in the liver or rarely in other internal organs, where they develop into metacestodes (larval stage). The basic structure, of a larval conglomerate, consists of a labyrinth of small chambers or microvesicles intermixed with host fibrous reaction and liver parenchyma, as depicted in Fig. 1B. The microvesicles are lined with an inner nucleated GL and a thin LL, which is much thinner than that observed in CE. The individual vesicles do not enlarge by expansion, but instead bud exogenously by breaking through the thin LL in a root-like fashion (Vogel, 1957, 1977, Mehlhorn et al., 1983, see also Chapter: Biology and Systematics of *Echinococcus* by Thompson, 2017). By repeating this process the vesicles proliferate unconstrained into an invasive, multichambered ('multilocular') lesion expanding into the liver parenchyma and beyond (Sato et al., 1993; Liu et al., 2014). This irregular structure can range in size from a few millimetres to a mass that occupies the entire liver. Macroscopically, this spongy lesion is a hallmark of 'alveolar' echinococcosis as this term best reflects its morphology. Rarely early vesicles may be visualized on US as haemangioma-like small lesions or MRI as small (approximately 3 mm in diameter) lesions of high-signal intensity on T2-weighted images (Aoki et al., 2015). Nodules of different sizes can develop. Those at the surface of the liver tend to be whitish, palpable and have a firm consistency. Central necrosis can occur with a cavity located within the lesions containing a viscous, yellowish fluid (amorphous eosinophilic cellular detritus, occasionally superinfected by bacteria). Large necrotic cavities (10–20 cm in diameter) can also develop. These AE pseudocysts are often misdiagnosed as CE. Viable metacestodes are growing and proliferating at the border to the sane liver tissue. In humans, brood capsules with protoscoleces are rarely formed, with a parasitological study finding protoscoleces in three of 20 human samples (Liance et al., 1990).

2.2.1.2 Natural course of metacestode growth

The initial phase of larval establishment is always asymptomatic. It may take years until the resulting lesions become apparent. An incubation period of 5–15 years is estimated for most cases (Sato et al., 1993; Ammann and Eckert, 1995; 1996). However, the infection can persist for decades,

remain unnoticed or might be detected incidentally through diagnostic imaging. The metacestode proliferates into the liver parenchyma. Lesions have necrotic zones and layers of histiocytes, fibroblasts, myofibroblasts and lymphocytes. In later phases, chronic inflammation occurs, often accompanied by a giant-cell foreign body reaction and fibrous tissue development. Calcifications or necrosis often occurs around degenerating parasitic vesicles (Fujioka et al., 1993). Unlike CE the metacestode is not demarcated at its outer limits by a fibrous, adventitial capsule (Eckert et al., 2011). Recently, staining of small particles of *E. multilocularis* (spems) outside the main lesion led to the suggestion that the parasite exerts systemic effects on the host (Barth et al., 2012). Deposits of these structures have been detected in the surrounding tissue, but also in the draining lymph nodes. It is theorized that spems may contribute to the spread of this cancer-like disease. If not treated, 90–100% of AE cases will die 10–15 years after diagnosis (Wilson et al., 1992).

Spontaneous deaths of the *E. multilocularis* metacestodes, in the human liver, were first documented in Alaska, the seminal work of Rausch et al. (1987). The lesions were referred to as having 'died out' and led to a spontaneous cure of these patients. Several cases, with such lesions, have been identified in mass screenings (Bresson-Hadni et al., 1994; Romig et al., 1999; Gottstein et al., 2001; Bartholomot et al., 2002). However, obtaining parasitological proof of a nonviable, 'dead' lesion has not been pursued due to ethical reasons. Spontaneous death of the parasite might actually occur quite regularly. Available data suggest that only 1–10% of egg-exposed persons will develop disease, with the majority eliminating the infection via innate and/or acquired immunity (Vuitton, 2003; Mejri et al., 2010; Brehm, 2010; Vuitton and Gottstein, 2010; see also Chapter: Immunology of Alveolar and Cystic Echinococcosis (AE and CE) by Gottstein et al., 2017).

2.2.1.3 Organ localization

AE can be regarded as a primary liver disease, as this organ is involved in all but 3% of the cases (Sato et al., 1993; Mesarina-Wicki cited in Ammann and Eckert, 1995; Kern et al., 2003; Piarroux et al., 2011). Initially the lesions are very small (a few millimetres in diameter) but they can grow more than 15–20 cm in diameter (Kern, 2010). In addition to liver infection, 34% of the cases suffer from manifestations in adjacent organs, such as diaphragm, perirenal tissue, abdominal lymph nodes and peritoneum, or more distant organs as illustrated in the European Registry Data presented in Table 3

Table 3 Sites of *Echinococcus multilocularis* metacestodes with single and multiple organ involvement

Organ involvement	PNM[a]	Number of cases (%)
Liver only	P1-4N0M0	351 (62.8)
Liver and other organs and tissues		190 (34.0)
Neighbouring organs	P1-4N1M0/1	
Diaphragm (n = 59)		
Extrahepatic vessels or ligaments (n = 38)		
Peritoneum (n = 33)		
Kidneys or adrenal glands (n = 26)		
Lungs or pleura (n = 15)		
Other sites (n = 35)		
Distant metastases	P1-4N0/1M1	
Lungs (n = 39)		
Brain (n = 17)		
Spleen(n = 10)		
Bones (n = 5)		
Other sites (n = 26)		
Extrahepatic sites only	P0N0/1M0/1	13 (2.3)
Spleen, peritoneum, lungs, vertebra, brain, kidneys, heart		
Data missing		5 (0.9)

Organ involvement at diagnosis in 559 patients from West and Central Europe.
P1 to 4, levels of liver involvement; *N*, lesions absent (0) or present (1) in neighbouring organs, *M*, metastases absent (0) or present (1).
[a]Kern et al. (2006).
Adapted from Kern, P., Bardonnet, K., Renner, E., Auer, H., Pawlowski, Z., Ammann, R.W., Vuitton, D.A., Kern, P., European Echinococcosis Registry., 2003. European echinococcosis registry: human alveolar echinococcosis, Europe, 1982–2000. Emerg. Infect. Dis. 9, 343–349.

(Kern et al., 2003). Only 3% of cases present primarily with an extrahepatic AE. The morphological structure of the metacestode, in other organs, is essentially the same as in the liver, but may differ slightly in certain localizations. Lung metastases often present as round to oval, dense and well-circumscribed nodules with an intersegmental distribution. Some lesions may contain an internal cavity. Retractions of adjacent organs such as pleura, bronchi and pulmonary vessels do not appear to occur with AE (Keutgens et al., 2013). If soft tissues are involved, lesions typically have a multivesicular morphology surrounded by a severe granulomatous inflammatory reaction. Protoscoleces or hooklets are rarely detected. Bone lesions present very heterogeneously, with active osteolysis detected on radiological examination. Needle biopsies demonstrate necrotic granulomatous lesions, suggestive

of tubercles. Brain lesions show a polycystic pattern with focal oedema (Schmid et al., 1998). Macroscopically the lesion consists of necrotic and fibrous tissue with numerous small cavities that take on a honeycomb appearance (Algros et al., 2003).

2.2.1.4 Staging classification

The tumour node metastases classification for malignant tumours, in particular hepatocellular cancer, guided the development of the WHO-IWGE classification system of AE (Kern et al., 2006). The WHO-IWGE classification scheme is used to describe the anatomical extent of AE and is based on the assessment and ranking of three components at the time of diagnosis: (P) location and extension of the primary (original) parasitic lesion within the liver; (N) involvement of neighbouring organs (whether or not the larva has spread to the nearby tissues/organs, including lymph nodes) and (M) presence or absence of metastases (whether or not the larva has spread to distant areas of the body, such as the lungs, brain, bones or any other location). As depicted in Table 4, the WHO-IWGE PNM-classification scheme stages lesions from I to IV based on the severity of disease. The impetus for developing the classification was to facilitate communication among clinicians, provide guidance for determining the most appropriate treatment strategy and to provide standardized information on the course and outcome of the disease (Kern et al., 2006; Brunetti et al., 2010). A clinical, single centre study corroborated the usefulness of the PNM classification, but a rather short median follow-up observation period of <5 years limited patient outcome assessment (Grüner et al., 2017).

2.2.2 Clinical features

After infection with *E. multilocularis*, there is always an asymptomatic incubation period of 5–15 years, except immunosuppressed patients (Chauchet et al., 2014). Most AE patients, in Western Europe, become symptomatic between the age of 50 and 60 years (Ammann and Eckert, 1996; Kern et al., 2003; Schweiger et al., 2007; Piarroux et al., 2011). Case series have not found a gender predominance, and cases in children are rarely reported. In contrast, in Asian countries, a diagnosis is often established in patients between 30 and 40 years of age, with more cases in females and young adults (Li et al., 2010; Usubalieva et al., 2013).

Initial clinical symptoms are either abdominal pain (mostly epigastric or right upper quadrant) or cholestasis with or without jaundice. In about

Table 4 PNM-classification of alveolar echinococcosis and assignment to stages I to IV

P	**Hepatic localization of the parasite**	
PX	Primary tumour cannot be assessed	
P0	No detectable tumour in the liver	
P1	Peripheral lesions	Without proximal vascular and/or biliary involvement
P2	Central lesions	With proximal vascular and/or biliary involvement of one lobe[a]
P3	Central lesions	With hilum vascular or biliary involvement of both lobes; and/or with involvement of two hepatic veins
P4	Any liver lesion	With extension along the vessels and the biliary tree
N	**Extra hepatic involvement of neighbouring organs**	
NX	Not evaluable	
N0	No regional involvement	
N1	Regional involvement of contiguous organs or tissues	Diaphragm, lung, pleura, pericardium, heart, gastric and duodenal wall, adrenal glands, peritoneum, retro-peritoneum, parietal wall (muscles, skin, bone), pancreas, regional lymph nodes, liver ligaments, kidney
M	**Absence or presence of distant metastasis**	
MX	Not completely evaluated	
M0	No metastasis	
M1	Metastasis	Lung, distant lymph nodes, spleen, CNS, orbit and eye, bone, skin, muscle, kidney, distant peritoneum and retroperitoneum
	Stage assignment	
I	P1N0M0	
II	P2N0M0	
IIIa	P3N0M0	
IIIb	P1-3N1M0, P4N0M0	
IV	P4N1M0, anyP anyN and/or M1	

P, levels of liver involvement, from low (P1) to high (P4) damage; *N*, lesions in neighbouring organs, *M*, metastases. Stage combines PNM levels with I to IIIa = localized disease, IIIb to IV = advanced disease. *X*, not assessed.

[a]Adapted from Kern, P., Wen, H., Sato, N., Vuitton, D.A., Gruener, B., Shao, Y., Delabrousse, E., Kratzer, W., Bresson-Hadni, S., 2006. WHO classification of alveolar echinococcosis: principles and application. Parasitol. Int. 55 (Suppl.), 283–287.

one-third of the cases, AE is diagnosed incidentally during a medical work-up for abnormal laboratory tests or symptoms and signs such as fatigue or hepatomegaly (Ammann and Eckert, 1996; Kern et al., 2003). AE symptoms are primarily dependent on location and secondarily on the size of the lesion (Bresson-Hadni et al., 2000). Centrally located hepatic lesions will present with cholestasis, jaundice and sometimes recurrent cholangitis, whereas lesion located in proximity to the hepatic veins and/or inferior vena cava will lead to a Budd—Chiari-like presentation with or without inferior vena cava obstruction. Parasite conglomerates in this location, often lead to metastatic lesions in the lungs, heart or other organs. Lesions located in the periphery of the liver remain asymptomatic for a long time and can become very large lesions prior to becoming symptomatic. Poor vascularization of these large lesions favours the development of large necrotic cavities, which are at high risk for secondary bacterial infection and/or abscess formation. The main causes of death, due to AE, are either septic shock, complications after major liver surgery, hepatic failure, cerebral AE or gastrointestinal bleeding due to secondary biliary cirrhosis (Bresson-Hadni et al., 2000).

2.2.3 Diagnostic imaging

2.2.3.1 Abdominal lesions

US and CT are the basic imaging techniques for evaluating patients with a potential diagnosis of AE. MRI is very often used to help differentiate AE from haemangiomas, especially if additional information regarding biliary or vascular structures is required prior to surgical intervention. In recent years, positron emission tomography (^{18}F-FDG-PET-CT) and contrast-enhanced ultrasound (CEUS) have been evaluated as possible tools to assess parasite viability.

Ultrasonography: With appropriate training, US is the initial imaging method of choice for the diagnosis of AE (Brunetti et al., 2010). Two typical sonographic patterns are observed in approximately 70% of the AE cases. The first pattern is characterized by irregular liver borders, which reflect the metacestodes invasive growth. Blurring is seen between the periparasitic granulomatous infiltrate and the normal liver, with hyper- (fibrous tissue) and hypoechogenic ('active' parasitic tissue) areas. The hyperechogenic fibrous tissue also contains also scattered calcifications, identified by the typical dorsal shadowing, as shown in Fig. 34. The second pattern shows a large hepatic lesion with central necrosis surrounded by a hyperechogenic ring corresponding to the parasitic fibrous tissue. Less

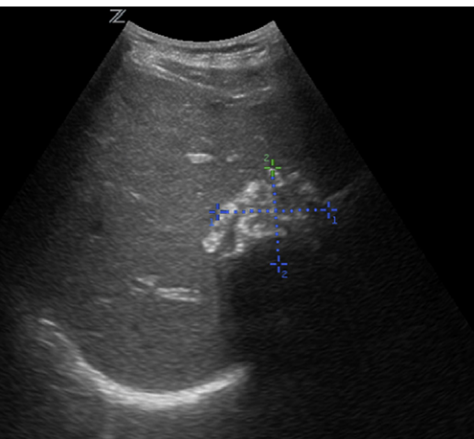
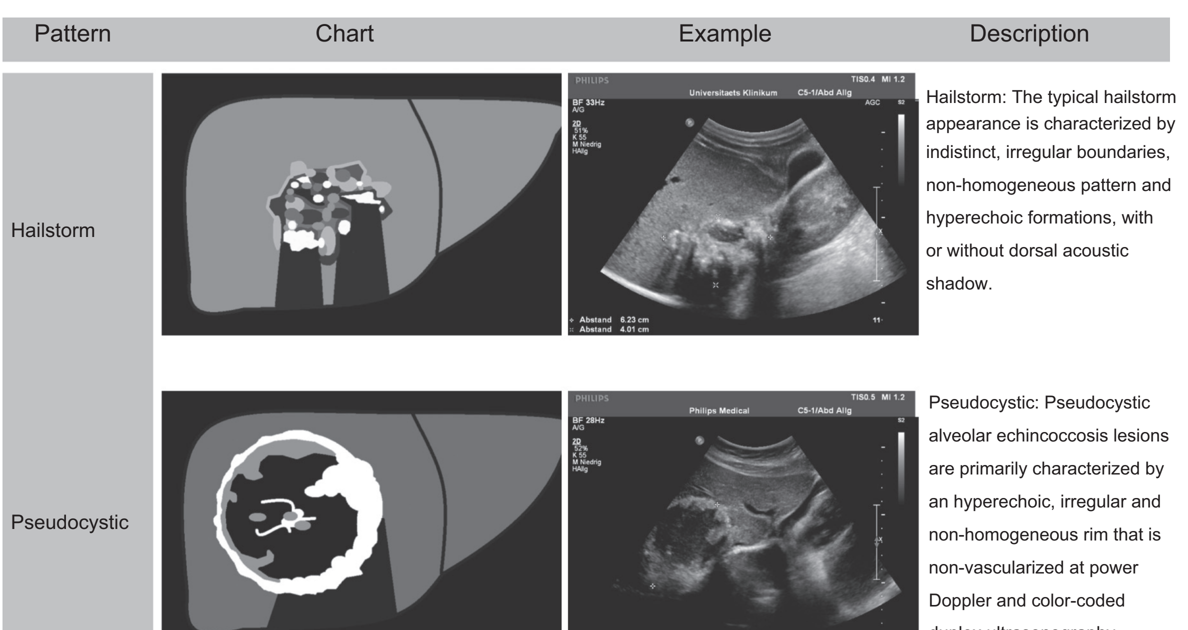


Figure 34 Abdominal ultrasonography. Hepatic alveolar echinococcosis lesion located in segment 4A. Lesion has a pseudosolid appearance with mixed echogenicity.

typical patterns are observed in the remaining third of cases. Small hyperechogenic nodules, corresponding to early AE lesions, can be misinterpreted as haemangiomas, or small calcified lesion may represent abortive or developing AE lesions (Bresson-Hadni et al., 2000; Bartholomot et al., 2002). US with colour Doppler is able to detect intrahepatic bile duct dilations and/or vascular obstruction of the hepatic vein, portal vein or the inferior vena cava (Kratzer et al., 2005). The role of contrast enhanced US to identify AE vitality requires further validation (Tao et al., 2011; Kaltenbach et al., 2015). Recently a proposed US classification was applied to AE cases retrospectively (Kratzer et al., 2015). As depicted in Fig. 35, assignment to five principal patterns was achieved by combining sonomorphology with type of calcification: (1) diffuse infiltrating; (2) primarily circumscribed tumour-like; (3) primarily cystoid; (4) small-cystoid/metastatic and (5) mainly calcified.

Computed tomography: CT is the best method to detect calcifications and number of lesions (Reuter et al., 2001). On CT, AE lesions are characterized by their irregular borders and a heterogeneous content with a combination of hyperdense scattered calcifications and of hypodense areas corresponding to necrosis and active parasitic tissue (Fig. 36A). After administration of intravenous contrast, there is typically no enhancement detected due to the poor vascularization of AE lesions (Fig. 36B). In contrast to US, CT is better able to further characterize calcified lesions. A completely calcified focus is compatible with a died-out lesion ('abortive AE') whereas



Pattern	Chart	Example	Description
Hailstorm			Hailstorm: The typical hailstorm appearance is characterized by indistinct, irregular boundaries, non-homogeneous pattern and hyperechoic formations, with or without dorsal acoustic shadow.
Pseudocystic			Pseudocystic: Pseudocystic alveolar echincoccosis lesions are primarily characterized by an hyperechoic, irregular and non-homogeneous rim that is non-vascularized at power Doppler and color-coded duplex ultrasonography.

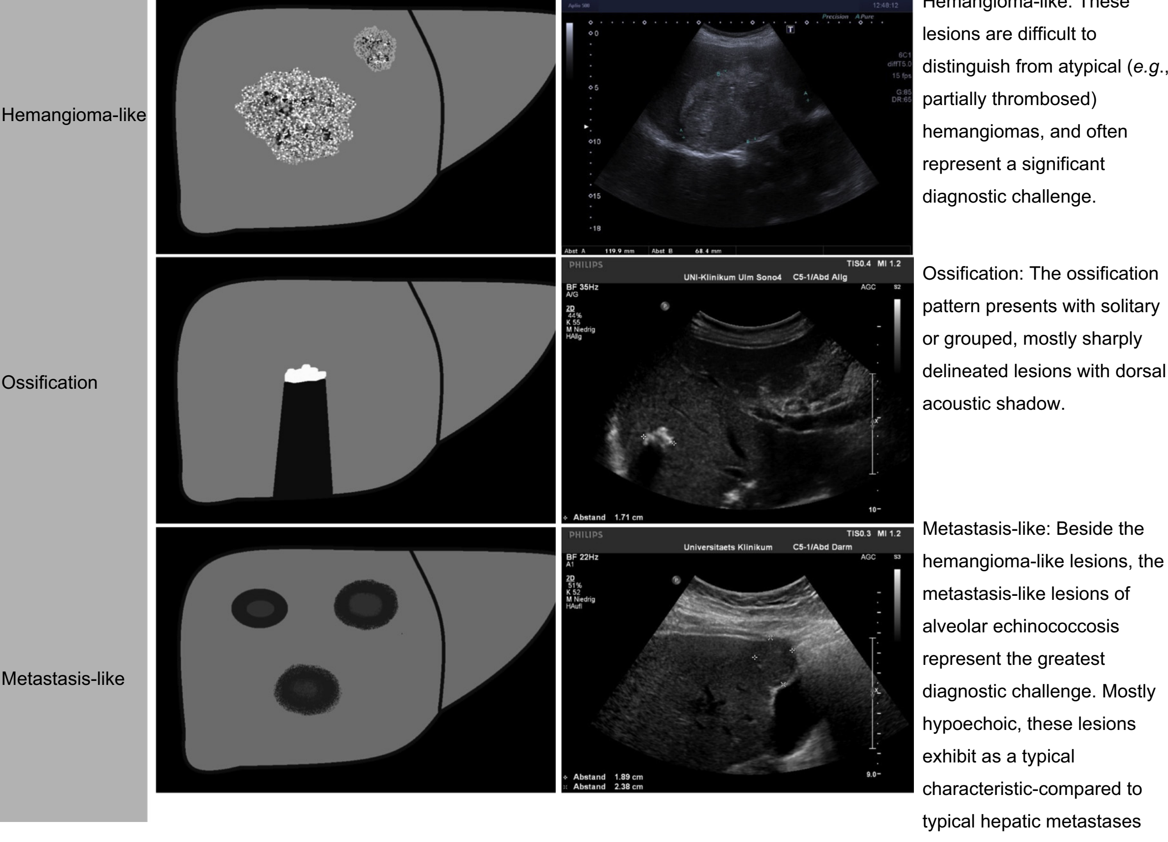


Figure 35 Alveolar echinococcosis. Description and classification of ultrasound images of the liver (*Echinococcus multilocularis* Ulm classification-ultrasound. *Adapted from Kratzer, W., Gruener, B., Kaltenbach, T.E., Ansari-Bitzenberger, S., Kern, P., Fuchs, M., Mason, R.A., Barth, T.F., Haenle, M.M., Hillenbrand, A., Oeztuerk, S., Graeter, T., 2015. Proposal of an ultrasonographic classification for hepatic alveolar echinococcosis:* Echinococcosis multilocularis *Ulm classification-ultrasound. World J. Gastroenterol. 21, 12392–12402.*

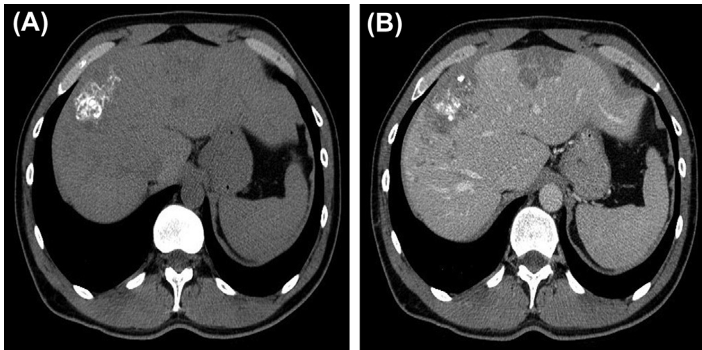


Figure 36 Alveolar echinococcosis. computed tomography scan of lesions in the right and left liver lobe. (A) Native image: Lesion in the right lobe with extensive calcifications. The lesion in the left lobe is barely visible as a hypoattenuated area. (B) Portal-venous phase of a contrast enhanced image: mass in the right and left lobe with no enhancement.

a calcified focus with a hypodense area rather suggests a small growing AE lesion (Bresson-Hadni et al., 2006).

Magnetic resonance imaging: T-2 weighted MRI with MRCP is the best method with which to visualize the honeycomb-like AE microcysts, which are considered pathognomonic for AE. MRI is also very useful to delineate the relationship between the AE lesion and biliary and/or vascular structures, although next generation CT images may also provide precise and useful information about the 'resectability' of a given lesion. Therefore both should be included in the preoperative assessment, especially if extensive resection or liver transplantation is being considered. AE lesions appear on MRI as heterogeneous tumours with irregular margins. On T2-weighted images, the lesions' microcysts appear as areas of high signal intensity. After contrast administration, these lesions typically show no contrast uptake (Fig. 37). Classification into five subgroups was proposed based on the MR findings, including the presence/absence of microcysts (Kodoma et al., 2003). This classification may prove useful in identifying metabolically inactive AE lesions. By comparing the MRI and ^{18}F-FDG-PET/CT findings, it was noted that type IV and V lesions (without microcysts) according to MRI exhibited no metabolic activity (Azizi et al., 2015). If these findings are confirmed by other studies, MRI may be another option by which to evaluate the metabolic activity and, thereby, vitality of AE lesions.

^{18}F-Fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET/CT): Conventional imaging techniques provide morphological information but

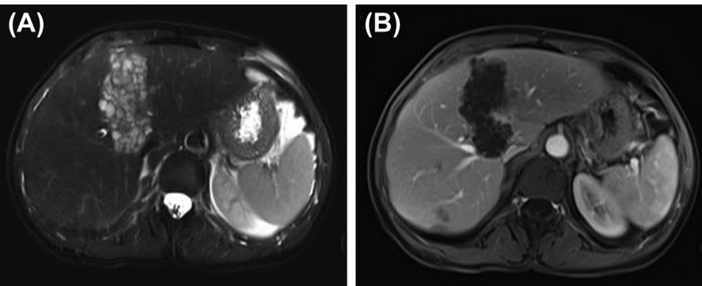


Figure 37 Magnetic resonance imaging scan of alveolar echinococcosis lesions in segment I and IV. (A) T2-weighted image showing multiple small cysts ('bunch of grapes'). (B) Portal venous phase of a contrast enhanced image: no contrast uptake by the alveolar echinococcosis lesion.

are unable to give information on parasite metabolic activity. Radio-labelled fluorodeoxyglucose ^{18}F-FDG-PET/CT is a newer technique to detect metabolically active lesions that metabolize glucose, such as malignant tumours or niduses of infections (Juweid et al., 2006: Stumpe et al., 2000). This imaging method has been evaluated in AE patients (Reuter et al., 1999, 2004; Stumpe et al., 2007). AE lesions typically exhibit a focally increased FDG uptake, forming localized hot spots in the lesion's periphery, whereas the centre of the lesions are usually PET negative, as shown in Fig. 38. In contrast to AE, CE lesions are typically PET negative (Stumpe et al., 2007; Niccoli Asabella et al., 2013). Follow-up ^{18}F-FDG-PET/CT examinations in patients undergoing long-term BMZ treatment revealed that, in some patients, the lesions became PET negative, implying that ^{18}F-FDG-PET/CT could potentially serve as a marker for AE vitality (see below)

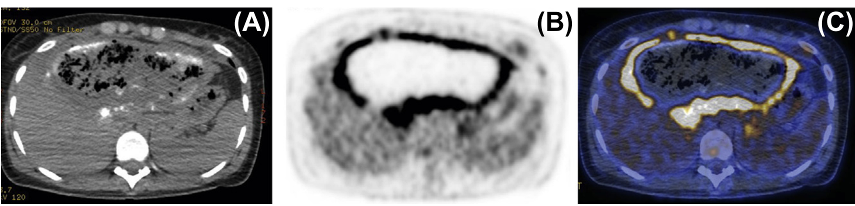


Figure 38 Alveolar echinococcosis. 18F-fluorodesoxyglucose-positron-emission-tomography/CT scan. (A) Native image of a large lesion in the right lobe with scattered calcification and a hypoattenuated center. (B) PET image showing an increased 18F-fluorodesoxyglucose uptake at the periphery of the lesion. (C) Fusion of 18F-fluorodesoxyglucose-positron-emission-tomography computed tomography images.

(Reuter et al., 1999, 2004; Stumpe et al., 2007). A recent study suggested that the accuracy of ^{18}F-FDG-PET/CT can be improved by the acquisition of late PET images (3 h after injection), which led to changes in the image interpretation in one-third of the patients (Caoduro et al., 2013).

Other imaging tools: Further evaluation of dual energy CT or spectral CT and DW-MRI is needed to validate their usefulness in assessing blood supply and/or metabolism of AE lesions (Liu et al., 2014).

2.2.3.2 Extrahepatic lesions

The lungs can be involved either by direct extension of the liver lesion or indirectly by parasitic metastasis. These lesions are usually multiple and can be located at the periphery of one or both lung lobes. They are typically small, solid and rarely have calcified foci (Kantarci et al., 2012). Brain esions are usually multilobular masses with peripheral calcifications and are surrounded by inflammatory oedema (Kantarci et al., 2012). AE lesions of the bones are characterized as focally invasive destructive processes without calcification (Ammann and Eckert, 1996). On CT or MRI, bone lesions appear as microvesicular structures (Kantarci et al., 2012). According to the WHO-IWGE consensus guidelines, initial radiological examination to exclude pulmonary and cerebral AE is recommended (Brunetti et al., 2010).

2.2.3.3 Differential diagnosis

Based on its invasive growth the main differential diagnoses of AE are primary and secondary hepatic malignancies, especially intrahepatic cholangiocarcinoma. If there is a maked cystic or pseudocystic component to the lesion, benign hepatic lesions such as cystadenomas and small CE cysts should also be considered. Small hyperechogenic lesions on US can be mistaken for a haemangioma. In the case of necrotic AE lesions, with a large central pseudocyst, the differential diagnosis also should include bacterial and amoebic abscesses.

2.2.4 Confirmation of diagnosis

A diagnosis of AE is based on clinical findings, epidemiological data, imaging techniques, histopathology and/or nucleic acid detection, and serology (Brunetti et al., 2010). Serological evaluation should follow a two-step approach (Siles-Lucas and Gottstein, 2001). The current status is presented in Chapter "Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals" by Siles-Lucas et al., 2017. In the first step,

diagnostically sensitive tests are employed (e.g., tests using *E. multilocularis* crude antigens). However, these tests lack specificity and cross-react with other helminth infections, including CE, as well as gastrointestinal malignancies and liver cirrhosis. In the second step, specific tests are used to confirm the results. Table 5 summarizes the criteria and rules to achieve a 'possible', 'probable' or 'confirmed' diagnosis of AE.

2.3 Clinical management

2.3.1 Treatment decision tree

AE is an invasive disease with many features resembling a malignant tumour including its poor prognosis if not treated appropriately. Therefore all patients with AE should be referred to a national/regional center, where expertise in extensive liver surgery, interventional radiology and endoscopy, radiological and serological diagnosis and medical treatment is available. The PNM-classification provides guidance to categorize individual patients. As surgery, in combination with two years of BMZ treatment is the only curative option, consultation with an experienced liver surgeon is mandatory. As discussed below, it is crucial to avoid a noncurative resection as most of the local complications can be treated by nonsurgical interventional techniques. The evaluation of a series of AE patients in Europe has provided strong evidence for the absence of benefit (and even the deleterious effect) of partial resections (Kadry et al., 2005; Buttenschoen et al., 2009b, 2009c; Piarroux et al., 2011).

2.3.1.1 Surgery

Radical surgical resection of the affected liver segments is the treatment of choice. Excision of the parasitic lesions should follow the rules of radical tumour surgery and requires a team with experience in advanced hepatobiliary surgery (Kadry et al., 2005; Buttenschoen et al., 2009c). The success rate for performing an extended hemihepatectomy has reached 95% for patients who were treated for hepatic metastases of colorectal cancer (Smith and D'Angelica, 2015). Earlier diagnosis and improved surgical techniques have led to 50% of AE lesions being considered resectable, up from 20% previously (Schweiger et al., 2007; Piarroux et al., 2011; Bresson-Hadni et al., 2000; Kadry et al., 2005). Uncontrolled studies provided the basis for the current WHO recommendation that patients should receive postoperative chemotherapy for 2 years after radical surgery (Ammann et al., 1990, 2004; WHO-IWGE, 1996; Brunetti et al., 2010). Recurrences have been reported more than 10 years after liver resection

Table 5 Diagnosis criteria of alveolar echinococcosis (AE)[a]

A) Clinical criteria	**1.** A slowly growing liver tumour (signs and symptoms vary with tumour location, size, and type). Associated symptoms: upper abdominal discomfort, inappetence, icterus, pruritus, weight loss. **2.** Incidental finding of an undefined liver tumour by imaging techniques in asymptomatic carriers or detected by screening strategies.
B) Epidemiological criteria	History of former/present residence in an endemic area
C) Diagnostic criteria	**1.** Suggestive organ lesion(s) for AE detected by imaging techniques [e.g., abdominal ultrasonography, computed tomography, magnetic resonance imaging (MRI)]. Lesions can be solid, partly multivesicular, with or without central necrosis, spotty calcifications) OR pathognomonic MRI images of 'bunch of grapes' with the detection of microcystic lesions of Kratzer classification of ultrasonography images[b] or Kodama classification of MR images[c]. **2.** Specific serum antibodies assessed by high-sensitivity serological tests, confirmed by a separate high specificity serological test. **3.** Histopathology compatible with alveolar echinococcosis or detection of *Echinococcus multilocularis* nucleic acid sequences in a clinical specimen.
D) Case definition and likelihood of AE diagnosis	'Possible' case: Any patient with a clinical or epidemiological history, and imaging findings compatible with AE, or serology positive for AE. 'Probable' case: Any patient with the combination of clinical history, epidemiological history, pathognomonic imaging findings and specific serology positive for AE on two tests. 'Confirmed' case: The above, plus either (1) histopathology compatible with AE and/or (2) detection of *E.multilocularis* nucleic acid sequence(s) in a clinical specimen.

[a]Adapted from WHO-Informal Working Group on Echinococcosis (Brunetti, E., Kern, P., Vuitton, D.A., Writing Panel for the WHO-IWGE, 2010. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 114, 1—16.).
[b]Kratzer et al. (2015).
[c]Kodama et al. (2003).

and 2 years of BMZ treatment. Therefore it is currently recommended to follow patients after curative resection for at least 10 years (Brunetti et al., 2010). Preoperative antiinfective therapy is not indicated if liver resection is planned at diagnosis. However, ABZ is often given for weeks to months after diagnosis if surgery is delayed. Long-term antiinfective therapy and/ or drainage of necrotic cavities may allow resections of lesions previously deemed inoperable. Nonradical (R1 or R2) resection should be avoided as the long-term survival of these patients is inferior to patients treated with long-term BMZ therapy (Kadry et al., 2005; Buttenschoen et al., 2009c).

2.3.1.2 Drug therapy

Treatment with BMZs was introduced into clinical practice in 1975 (Schantz et al., 1982; Müller et al., 1982; Kern, 1983; Davis et al., 1986). Unlike for CE, BMZs only suppress *E. multilocularis* growth, therefore necessitating long-term treatment (Eckert, 1986). As for CE the active antiparasitic compounds are MBZ itself and the main metabolite of ABZ, albendazole sulfoxide. Initially only MBZ was available in many parts of the world. However, in recent years, most centres have used ABZ because its better bioavailability allows twice daily dosing. The usual dose of ABZ is 10—15 mg/kg/day given in two daily doses. To ensure adequate intestinal absorption the drug has to be taken with a fat-rich meal. For most patients 2 × 400 mg daily is an appropriate starting dose. Continuous ABZ treatment is well tolerated and has been used for an extended period of time in some patients. As for CE, intermittent treatment should no longer be used. In rare instances the dose of ABZ has been increased to 20 mg/kg/day (Brunetti et al., 2010). If ABZ is not available or not tolerated, MBZ is still a good alternative. The dose of MBZ is 40—50 mg/kg/day split into three doses which are also given with fat-rich meals.

Monitoring of blood drug levels is indicated to confirm adherence to treatment, to ensure adequate therapeutic drug levels and to avoid toxic reactions (see below). BMZs are embryotoxic and teratogenic. Therefore the use of these drugs should be avoided in pregnancy, and contraceptive measures are mandatory for women of reproductive age. As the treatment must be undertaken for years a decision to temporarily stop treatment (structured interruption) can be taken after months or years (Reuter et al., 2004; Ammann et al., 2015; Grüner et al., 2017). In this case the absence of progressive disease needs to be verified. BMZs are usually well

tolerated in 70–80% of cases, but more adverse side effects are seen in patients with immunosuppression (Chauchet et al., 2014). The most common side effects are: elevation of transaminases, proteinuria, transient hair loss, gastrointestinal disturbances, leukopenia and neurologic symptoms, including sleeplessness and vertigo.

BMZ treatment is recommended for 2 years after complete resection of AE lesions. Long-term medical treatment is indicated in cases of inoperable AE, after liver transplantation and after incomplete lesion resection. It is difficult to assess the effectiveness of long term BMZ treatment. Most commonly, effectiveness is assessed by using CT or other imaging method to measure the larval mass. Since BMZ treatment is largely considered nonparasitocidal, regression and nonprogression are usually considered treatment success. Whether long-term BMZ treatment eventually exerts an effect on parasite viability is still under debate, although the evidence for such an effect is mounting (Wilson et al., 1992; Azizi et al., 2015; Ammann et al., 2015). Early experimental animal data showed that treatment with a BMZ was parasitostatic but did not kill the AE metacestodes (Eckert and Pohlenz, 1976; Schantz et al., 1982). Similar data have been obtained after parasite tissue from patients treated with MBZ was transplanted into animals (Ammann and Eckert, 1996). The first report suggesting a parasitocidal effect in vitro and in vivo was already reported in 1992 (Wilson et al., 1992). This potential parasitocidal effect has been documented in patients with disseminated disease (Caoduro et al., 2013; Bardonnet et al., 2013; Ammann et al., 2015) and/or associated immunosuppression (Bresson-Hadni et al., 2011). Whether the long-term efficacy of BMZ, in some patients with AE, is related to direct parasitocidal activity or an indirect effect through immune stimulation is unknown (see Chapter: Immunology of Alveolar and Cystic Echinococcosis (AE and CE) by Gottstein et al., 2017).

2.3.1.3 Adjunct interventional treatment

A number of local complications occur in patients with nonresectable AE for which interventional procedures are the best option (Bresson-Hadni et al., 2006). Biliary complications are reported in 10–44% of the patients with nonresectable AE with long-term follow-up (Bresson-Hadni et al., 2006; Frei et al., 2014; Graeter et al., 2015). Percutaneous or endoscopic interventions can be used successfully to treat cholangitis, liver abscesses and jaundice with biliary strictures and have replaced palliative surgery. Repeated stenting using multiple plastic stents may alleviate acute

biliary complications and allow long-term biliary drainage even in patients with tight intrahepatic bile duct obstruction (Ambregna et al., 2017) Peri-interventional antibiotic prophylaxis and extensive lavage of the bile ducts before stenting in patients undergoing ERCP, is recommended to avoid bacterial infection of necrotic cavities and the biliary tree. The role of ursodeoxycholic acid has not been extensively studied but is recommended by some experts (Brunetti et al., 2010; reviewed by Tamarozzi et al., 2014c). Rarely stenting of the hepatic veins has been employed to treat Budd–Chiari syndrome due to AE (Vogel et al., 1996).

2.3.1.4 Rescue transplantation

Liver transplantation was first introduced as an option for patients with nonresectable AE in 1986. The first European multicenter series of 44 AE cases treated with orthotopic liver transplantation (OLT) reported a 5-year survival rate of 71% and a 5-year disease-free of 50% (Koch et al., 2003). Series with shorter follow-up periods were also reported from China and Turkey, with an overall survival rate between 60% and 80% (Pan et al., 2004; Xia et al., 2005; Aydinli et al., 2015; Ozdemir et al., 2015; Li et al., 2007). An inherent problem associated with OLT is AE recurrence (Bresson-Hadni et al., 1999) (Table 6). Therefore BMZ treatment pre- and post-OLT as well as low level immunosuppression are strongly recommended (Koch et al., 2003). Considering the good outcome associated with medical treatment of nonresectable AE lesions, OLT should only be considered in patients nonresponsive to medical treatment. These patients include those with outflow problems (Budd–Chiari syndrome) or recurrent life-threatening cholangitis with secondary biliary cirrhosis. Extrahepatic disease is no longer an absolute contraindication for OLT in cases where disease manifestations can be well controlled with a BMZ (Bresson-Hadni et al., 2011). Recently a liver autotransplantation as an alternative technique has been developed by Chinese surgeons (Wen et al., 2011), where the liver is removed, the parasitic lesions are resected ex vivo and the remaining liver returned to the patient (autotransplantation). In this case the immunosuppressive drug regime associated with OLT can be avoided. This technique was used as a rescue treatment for large lesions with vascular involvement. Midterm evaluation of a series of 15 cases shows promising results (Wen et al., 2016). However, long-term evaluation is needed to assess the viability of autotransplanted liver (Mantion and Vuitton, 2011).

Table 6 Results of liver transplantation in patients with alveolar echinococcosis

References	Year of publication	Number of pts	Median Age Yrs. (range)	Type of transplant DDLT/LDLT	Median Follow-up after OLT months (range)	Survival %	BMZ treat-ment	Recurrence n
Koch et al. (2003)	2003	44	48 (16—67)	44/0	71 (0—132)	55	23/44	7/44 (16%)
Li et al. (2007)	2007	7	41 (16—58)	7/0	n.r (0—68)	71	yes	0
Aydinli et al. (2015)	2015	27	39 (13—65)	7/20	15 (0—39)	78	For 2 yrs after OLT	1/27 (4%)
Ozdemir et al. (2015)	2015	10	35 (19—61	0/10	15 (2—54)	70	no	3/10 (30%)

BMZ, benzimidazoles; *DDLT/LDLT*, deceased donor liver transplant/living donor liver transplant; *OLT*, orthotopic liver transplantation.

2.3.2 Follow-up management

After initiation of any type of treatment, long-term follow-up by US at 6–12 month intervals and by CT and/or MRI at 1–3 years' intervals is recommended. Disease progression is defined by enlargement of lesions over time. Even after curative resection and 2 years of treatment with a BMZ, follow-up for at least 10 years is recommended, as late recurrences have been reported (Ammann et al., 2004). After curative resection there is usually a sharp decline in anti-EmII/3-10 (anti-Em18) and anti-Em2 levels with normalization (i.e., negative serology) in most patients within 3 years (Ammann et al., 2004; Tappe et al., 2009a). Interpretation of serological results in nonresectable patients treated with a BMZ is less clear, but in approximately 60% of these patients, normalization of anti-EmII/3-10 (anti-Em18) levels are observed (Ammann et al., 2015). As discussed above, in the majority of patients, AE lesions lost their metabolic activity on ^{18}F-FDG-PET/CT during long-term BMZ treatment (Reuter et al., 2004; Stumpe et al., 2007). However, this loss of biological activity was insufficient to identify patients who might be candidates for cessation of treatment with a BMZ (Reuter et al., 2004). More recently two different groups reported that the combination of anti-EmII/3-10(anti-Em18) or Em2 PLUS serology and ^{18}F-FDG-PET/CT could successfully identify patients who could stop BMZ treatment (Table 7) (Caoduro et al., 2013; Ammann et al., 2015). Delayed image acquisition (3 h) after FDG injection has been proposed to make ^{18}F-FDG-PET/CT more sensitive and thus avoid false negative images at the time of diagnosis and during follow-up (Caoduro et al., 2013). As data are still limited, this combined approach, along with delayed image acquisition requires further validation.

E. multilocularis metacestode stem cells (Brehm and Koziol, 2014) have the potential, as do cancer cells, to reinitiate AE lesions in the liver or other organs, even years after apparently successful treatment (Joliat et al., 2015). Thus all patients with AE need to have a regular follow-up (every 3 months, then 6 months, then yearly, depending on the clinical status and the presence of complications). The follow-up should include US and serology, blood cell count and aminotransferase levels, and ideally ^{18}F-FDG-PET during BMZ treatment. Yearly follow-up should be maintained for at least 5 years after cessation of BMZ therapy (alone or postsurgery).

Patients treated with a BMZ require regular follow-up visits to evaluate liver values and blood cell count. These visits should occur after 1, 4 and 12 weeks after starting treatment and then every 6 months if no complication

Table 7 Alveolar echinococcosis: treatment interruption based on serology results and ^{18}F-fluorodesoxyglucose-positron-emission-tomography/computed tomography

Reference	Year of publication	Number of patients	Treatment duration before stopping treatment (years)	^{18}F-FDG-PET/CT	Serology	Treatment duration before stopping treatment mo. median (range)	Number of pts. that stopped treatment(%)	Follow-up after stopping treatment mo. months mean/median(range)	Recurrence
Caoduro et al. (2013)	2014	44	≥2	Delayed image acquisition	Em2 plus	n.a.	7 (16%)	23 (8–37)	0
Ammann et al. (2015)	2015	34	≥2	Conventional image acquisition	EmII/3-10	50 (34–276)	11 (32%)	70 (16–82)	0

^{18}F-FDG-PET/CT = ^{18}F-fluorodesoxyglucose- positron-emission-tomography/computed tomography.

arise. As compliance is crucial to ensure treatment success and to avoid drug toxicity, determination of ABZ sulfoxide blood levels, 4 h after the morning dose, is recommended 1, 4 and 12 weeks after starting treatment, 2–4 weeks after each dose adjustment (Brunetti et al., 2010), and whenever unexpected events (e.g., changes in imaging features, raised antibody titres, etc.) are observed at follow-up. Monitoring of ABZ sulfoxide also helps to evaluate patient adherence to treatment and can be used to adjust ABZ dosage in case of apparent resistance to treatment or if adverse effects occur (Vuitton and Bresson-Hadni, 2014). The therapeutic range of ABZ is between 0.65 and 3 μmol/L. It is recommended to reduce the ABZ dose if two sequential measurements are above 10 μmol/L. For MBZ, plasma levels should be over 250 nmol/L (Bresson-Hadni et al., 2000). In case of life-threatening side effects, a switch to the alternative BMZ is sometimes possible.

2.3.3 Failure management

Other drugs: As discussed above, BMZ failure is rare, and no pharmacological drug resistance has ever been reported. However, individual resistance to treatment may be observed, with transaminase consistently five times greater than normal value and/or severe leucopenia the most common reasons for treatment cessation. In such cases, therapeutic options are limited (Hemphill et al., 2014). A number of different drugs have been evaluated either in vitro [PZQ, amphotericin B (AMB), alpha-difluoromethylornithine, artemether, caspofungin, itraconazole (ITZ), ivermectin, mefloquine, methiazole (MTZ), miltefosine, nitazoxanide (NTZ), rifampin, and trimethoprim–sulfamethoxazole] or in vivo (NTZ, AMB) (Reuter et al., 2003; Küster et al., 2011, 2013, 2014; reviewed by Vuitton and Bresson-Hadni, 2014). Some of them (ABZ, ITZ, MTZ and NTZ) showed promising in vitro activity (Reuter et al., 2006, 2010). NTZ, the most promising compound based on in vitro results, failed to exhibit a clinically meaningful effect in humans (Kern et al., 2008; Tappe et al., 2009b). AMB, on the other hand, has shown a parasitostatic effect both in vitro and in vivo (Reuter et al., 2003). However, the number of patients treated thus far is limited. The drug also has significant side effects and must be administrated intravenously. AMB may be an option in the rare cases where BMZ treatment fails. Hopefully the recent elucidation of the *E. multilocularis* genome will lead to the identification of new targets in the life cycle of this parasite that can be blocked with small molecules and the discovery of new medical treatment options (Brehm and Koziol, 2014).

2.3.4 Confounding conditions

Effects of immunosuppression on *E. multilocularis* growth have been extensively studied in experimental animals (reviewed by Vuitton and Gottstein, 2010). The first evidence of the facilitating effect of immunosuppression on AE lesion development in humans came from studying patients who received a liver transplant (Koch et al., 2003). Fast progression of the lesions in a patient with AIDS (Sailer et al., 1997) and, conversely, the protective effect of anti-HIV treatment in another patient (Zingg et al., 2004) confirmed the role of immunosuppression in patients with AE. In AE patients treated by liver transplantation, appearance or growth of lung, brain or spleen metastases as well as early or late reinfection of the transplanted liver by *E. multilocularis* have been observed (Koch et al., 2003). Liver US images, observed in these patients, confirmed that early AE may look like liver haemangiomas, as was previously observed at mass screenings in hyperendemic areas (Bartholomot et al., 2002). MRI, of these patients, shows the typical microcysts of AE. Such observations support the recommendation to reserve liver allotransplantation for very advanced cases and to perform a complete evaluation of the disease by CT scan and MRI before transplantation (Brunetti et al., 2010).

Since the beginning of the 21st century, a number of AE cases have been reported in patients who received other kinds of organ transplants or who were treated for malignant or chronic inflammatory diseases (Gruener et al., 2008; Kayacan et al., 2008; Gaultier et al., 2009; Geyer et al., 2011; Weiner et al., 2011; Kern et al., 2011; Dentan et al., 2012). The increase in the occurrence of AE in such patients was evidenced in a study using the data from the French AE registry database (1982–2012) (Chauchet et al., 2014). In this study, out of 509 AE cases diagnosed from 1982 to 2012, 50 patients were found with such immunosuppression-associated conditions before or at AE diagnosis. The number of such patients has also increased in the last 2 decades (1993–2002 versus 2003–2012). In decreasing order, underlying diseases include malignant diseases, chronic inflammatory diseases, transplantation (heart, kidney) and AIDS. Whatever the condition, acquired therapeutic immunosuppression appears to be the main factor for the occurrence of AE and its fast progression in the patients. In the above mentioned study, among patients diagnosed in the last decade (2003–2012), 38/42 received an immunosuppressive regimen. In 9 cases, two immunosuppression-associated conditions were diagnosed in the same patient.

The preexisting condition and associated therapy can modify how AE presents. For example, these patients may have an acute presentation that mimics a pyogenic abscess, both clinically and at imaging (Weiner et al., 2011; Chauchet et al., 2014). Faster metacestode growth than in patients without immunosuppression is suggested (Chauchet et al., 2014). Negative serology (observed in 10% of patients) also adds to the delay in diagnosing AE, in such patients, and may contribute to an erroneous diagnosis and the wrong therapeutic interventions. It is likely that the number of patients with underlying immunosuppression-associated conditions and incidental AE-detection will increase in Europe and in other endemic areas, such as China.

2.4 Outcome and prognosis

Therapeutic management of AE patients clearly requires a multidisciplinary approach, in which BMZ therapy is a common denominator. In a literature review the AE treatment success rate in 19 studies was reported to range between 55% and 100%, with a success rate above 70% in all but one study (Reuter et al., 2000). Long-term BMZ treatment can result in biliary complications, which can impact patient survival (Frei et al., 2014; Wilson et al., 1995). These complications usually develop after more than 10 years and increase when a noncurative surgery was performed (Frei et al., 2014). A complete evaluation of the disease process (including thoracic and brain CT) is necessary before any therapeutic decision and to serve as a basis for follow-up. Outcome will depend on the size of the lesion(s), its location in the liver, vascular and biliary involvement, the presence or absence of bacterial infection, involvement of adjacent organs, and the presence or absence of distant metastases. Use of the PNM classification can help to determine a patient's prognosis (Kern et al., 2006, Grüner et al., 2017).

In Europe and Japan, earlier diagnosis and better management of AE cases has resulted in an improved prognosis for patients. A survival analysis from Switzerland demonstrated that recently diagnosed AE patients have a life expectancy that is only shortened by about 3 years compared to the general Swiss population. In contrast, an AE patient diagnosed in 1970 had a life expectancy that was reduced by 18–21 years (Torgerson et al., 2008). The reasons for this improved prognosis are multifactorial, including the introduction of treatment with BMZs, better surgical techniques and increased availability of multidisciplinary treatment centres. In France, life expectancy of AE patients 1 year after diagnosis is similar to

that of their fellow citizens without AE (Piarroux et al., 2011). Globally the observed survival of 347 AE patients (2742 person-years) was lower than the expected survival of the general population matched for sex, age and calendar year ($p < .001$). However, the baseline excess mortality hazard decreased steeply during the first 2 years and remained close to that of the general population until 5 years postdiagnosis. A poor prognosis was associated with older age and invasion of the hilar region of the liver, with associated biliary complications. Conversely, medical treatment with BMZs (with or without surgery) was associated with a better survival (Piarroux et al., 2011). In recent cohort studies following AE patients treated with a BMZ, the 10- and 15-year survival rates were 80–83% and 53–80%, respectively. This is in contrast to a 0–25% 10-year survival rate in the pre-BMZ area (Wilson et al., 1992; Bresson-Hadni et al., 2000; Caoduro et al., 2013; Grüner et al., 2017). AE is still considered a lethal disease for symptomatic patients who live in many endemic areas, including parts of central Asia and China. For example, a 25% case fatality rate was reported in a recent case series of AE patients receiving palliative surgery (Ayifuhan et al., 2012).

2.5 Burden of alveolar echinococcosis

Assessment of the burden of AE faces many of the same challenges as for CE. However, since livestock are not part of the *E. multilocularis* life cycle, burden estimates tend to focus solely on human losses. As with CE the DALYs is the most commonly used metric to assess the nonmonetary burden of AE on human populations. The first global estimate of the burden of AE was published in 2010 (Torgerson et al., 2010). This study calculated that there were approximately 18,200 new cases of AE per year, with 91% of these cases occurring in China. Based on these values, a median of 666,434 DALYs lost per year was estimated. The WHO- Foodborne Disease Burden Epidemiology Reference Group (FERG) also published global AE burden estimates for the year 2010 (Torgerson et al., 2015). In general the global burden of CE appears to be greater than for AE due to higher overall disease frequency. However, on an individual patient level, burden tends to be higher for AE sufferers due to the clinical severity of the disease. As of yet the Global Burden of Disease (GBD) Study has not elected to include AE in their estimates independent from CE (Murray et al., 2012).

There have only been a few regional studies looking at the nonmonetary and monetary burden of AE. As with CE the first of which was conducted for a highly endemic pastoralist population located in western Sichuan

Province, China (Budke et al., 2004). Since this initial study, there have been only a few attempts to evaluate the burden of AE in specific geographic locations, such as in patient seeking treatment in Switzerland (Torgerson et al., 2008). Data gaps for estimating the burden of AE are similar to those encountered for estimating the monetary and nonmonetary burden of human CE and include a lack of frequency data and information on clinical course in areas with poor medical infrastructure.

3. NEOTROPICAL ECHINOCOCCOSIS

3.1 Introduction

Neotropical echinococcosis (NE) is found in tropical areas of Central and South America. In 1972 Rausch and Bernstein described the new species, *Echinococcus vogeli*, after collecting an adult specimen from a bush dog (*Speothos venaticus*) captured in Ecuador (Rausch and Bernstein, 1972). The authors predicted that the parasite's larvae developed in pacas (*Cuniculus paca*), which are large rodents and the preferred prey of the bush dog. This prediction was proven correct (D'Alessandro et al., 1979; Vizcaychipi et al., 2013). Experimental infections have helped to confirm the *E. vogeli* life cycle and parasitological studies have illustrated the morphological differences in the shape and size of *E. vogeli*'s rostellar hooks compared to other *Echinococcus* species (reviewed by Tappe et al., 2008). In humans, infection with the metacestode stage of *E. vogeli* causes polycystic NE (D'Alessandro et al., 1979, 1981). The clinical picture of human *E.vogeli* infection is complex and, if untreated, the disease is considered fatal (D'Alessandro and Rausch, 2008; Eckert et al., 2011).

The second species causing NE in humans is *Echinococcus oligarthrus* (D'Alessandro and Rausch, 2008). The known distribution, of this parasite, ranges from Costa Rica to the La Pampa region of Argentina. In humans, infection with the metacestode stage of *E. oligarthrus* causes unicystic NE (D'Alessandro and Rausch, 2008; Eckert et al., 2011). Thus far, only four human cases of unicystic NE have been reported in the literature (D'Alessandro and Rausch, 2008; Soares et al., 2013). However, additional cases of *E. oligarthrus* infection are anticipated now that genotyping methods are available to better differentiate infection with *E. oligarthrus* from other *Echinococcus* spp. NE is considered as a possible emerging zoonotic disease in rural areas of Central and South America (D'Alessandro, 1997).

3.2 Clinical diagnosis and definitions

3.2.1 Metacestodes in humans

Of the two neotropical species, *E. vogeli* is believed to be clinically more severe. Metacestodes multiply asexually by endogenous and exogenous proliferations, invade the affected organs, and can metastasize to distant organs (Rausch and D'Alessandro, 1999). Thus the growth characteristics resemble most likely those described for *E. multilocularis*, as depicted in Fig. 1B. These polycystic lesions can be visible on the surface of the liver, while extending into the liver parenchyma and sometimes along the bile ducts as shown in Fig. 39. Metacestodes can also spread to the peritoneal cavity, pericardium, lungs, pleura, superior vena cava and right atrium. Clusters of cysts vary from 10 mm in diameter to masses replacing almost the entire liver, with individual cysts varying in size from 5 to 80 mm in diameter. The typical colour appears as yellowish with gelatinous content. Some cysts may become necrotic, or calcify. As with other forms of echinococcosis, metacestode development is a chronic process. In contrast to *E. vogeli*, *E. oligarthrus* infections present as a single lesion in a unique anatomical region such as the orbit of the eye, the myocardium or in the liver. Cysts tend to be small

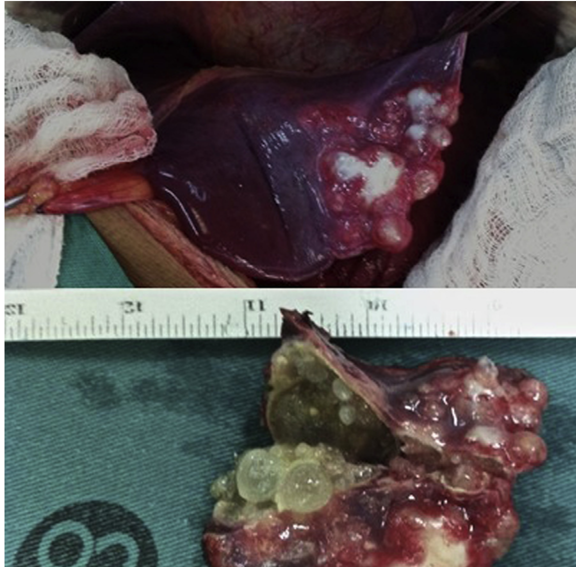


Figure 39 *Echinococcus vogeli* polycystic metacestode affecting the left lateral segment of the liver. *Courtesy by Nilton Siqueira.*

(15–30 mm in diameter) upon detection. If multiple cysts are found, they are usually located in separate locations.

3.2.2 Clinical features

Clinical features, associated with NE, are dependent on the location and size of the cyst(s). To date, 242 cases of polycystic NE are documented (February 2016), with clinical data available for 78 patients with complete medical histories (D'Alessandro and Rausch, 2008; D'Alessandro, 2010). Of these patients, 60 patients have been followed for more than 10 years (Siqueira et al., 2013). Polycystic NE is typically diagnosed in individuals between 40 and 60 years of age, which may reflect a long latent period. However, acute clinical manifestations can also occur. Extensive lesions are rare in patients under 22 years of age. The disease is identified in men 1.5 times more often than in women. The liver is the organ most frequently involved (approximately 80% of cases), followed by the lungs, peritoneal cavity and other anatomic regions as can be reported in Table 8. A key diagnostic feature of polycystic NE is peripheral calcification of the cysts, which occurs in approximately 96% of cases (Fig. 40) (D'Alessandro et al., 1981; D'Alessandro, 2010; Siqueira et al., 2013).

Table 8 Frequency of clinical features of neotropical echinococcosis (NE)

Clinical presentation[a]	n	%
Polycystic neotropical echinococcosis		
Cysts in the liver only	117	80.1
Liver + abdominal cavity	66	56.4
Liver, abdominal cavity + hepatic failure	37	31.6
Liver + lung/thorax	14	12.0
Cysts in lung only	11	7.5
Cysts in mediastinum only	13	8.9
Cysts in retroperitoneum only	1	0.7
Unicystic NE		
Cyst in orbit only	2	1.4
Cyst in heart only	1	0.7
Cyst in liver only	1	0.7
Total	**146**	**100.0**

[a]Adapted from D'Alessandro, A., Rausch, R.L., 2008. New aspect of neotropical (*Echinococcus vogeli*) and unycystic (*Echinococcus oligarthrus*) echinococcosis. Clin. Microbiol. Rev. 21, 380–401; D'Alessandro, A., 2010. Actualización. Hidatidosis poliquística tropical por *Echinococcus vogeli*. Rev. Asoc. Med. Argent. 123, 16–23; Siqueira, N.G., de Siqueira C.M., Rodrigues-Silva, R., Soares Mdo, C.P., Póvoa, M.M., 2013. Polycystic echinococcosis in the state of Acre, Brazil: contribution to patient diagnosis, treatment and prognosis. Mem. Inst. Oswaldo Cruz 108, 533–540.

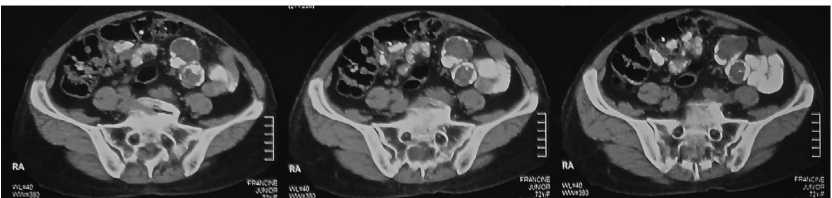


Figure 40 Polycystic neotropical echinococcosis disseminated the peritoneal cavity. computed tomography scan shows cystic lesions with peripheral calcification. *Courtesy by Nilton Siqueira.*

The most common presenting clinical manifestation, of polycystic NE, is the upper abdominal pain. An abdominal mass, with irregular surfaces, can often be palpated in the right upper quadrant. The mass usually moves with respiration and the site is tender to touch. Patients may also present with advanced hepatic disease, including obstructive jaundice. Liver damage appears clinically similar to hepatic cirrhosis, and patients may present with portal hypertension, jaundice, haematemesis or combinations thereof. Cases with pulmonary NE may present with coughing and production of purulent sputum. These patients may also have fever and chills along with abdominal symptoms. Polycystic NE has also resulted in cardiomegaly, congestive heart failure and/or pulmonary oedema.

As shown in Table 8, two of the four known human cases of unicystic NE presented with orbital retro-ocular proptosis, eyelid ptosis, headache and blindness (D'Alessandro and Rausch, 2008; Soares et al., 2013).

3.2.3 Imaging procedures

Abdominal US is the imaging method of choice for epidemiological surveys to detect polycystic NE. Images show single or multiple round and anechoic cysts with thin walls. Multiple lesions may involve several liver segments. A plain radiograph demonstrating polycystic masses with small calcifications is suggestive of polycystic NE. Calcifications are ring-shaped and tend to be 20–30 mm in diameter, with a dense halo and a clear centre (D'Alessandro, 1997; D'Alessandro and Rausch, 2008). Abdominal CT has been used to diagnose polycystic NE. On CT, polycystic NE appears as multiple, round and hypodense structures of varying sizes. The appearance of calcifications on CT can also help confirm a diagnosis (Fig. 41). US and CT can also be used to monitor the evolution of disease.

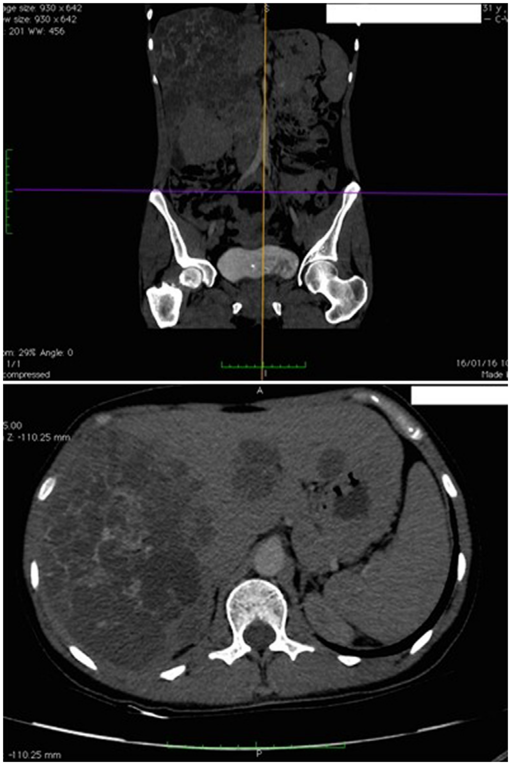


Figure 41 Polycystic neotropical echinococcosis. computed tomography scan of a female patient, 31-years-old, with severe hepatic lesion: multiple cystic lesions with peripheral calcifications. *Courtesy by Nilton Siqueira.*

3.2.4 Diagnosis

The clinical diagnosis of NE is based on patient history and physical examination, imaging studies, laboratory tests (parasitological, histopathological and molecular). It is important to ask questions regarding possible risk factors for NE (e.g., history of hunting pacas and interaction with bush dogs and dogs) as well as obtain an extensive travel history. Most identified cases of NE have lived in rural areas. Immigration status and international travel history should also to be taken into account when developing a differential diagnosis for abdominal masses. Care should be taken to differentiate infection with *E. vogeli* and infection with the more common, *E. granulosus* (D'Alessandro, 1997; Stijnis et al., 2015).

Laboratory tests cannot be used to make a definite diagnosis of polycystic NE, but they are important in assessing damage to the biliary tree. For

example, alkaline phosphatase, bilirubin, hepatic transaminases and gamma globulin will be elevated, whereas albumin and haemoglobin will be decreased with biliary damage. Eosinophilia is not common with polycystic NE and a high eosinophil count, in a polycystic NE patient, may be due to coinfection with other parasites. Blood cell counts and liver function tests are used to monitor therapy with ABZ.

There are no specific serological tools available for NE (see also Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017). Serological testing may be performed with hydatid fluid antigens used to diagnose CE [e.g., indirect haemagglutination, and more specifically, ELISA or immunoblot (Western blot)]. These tests do not have perfect sensitivity or specificity and cross-reaction with *Taenia solium* cysticercosis is possible. Therefore serological tests should be used and interpreted in association with the epidemiological data, clinical manifestations and imaging findings. Suspect cases of NE should be referred for higher level diagnostics and care. Diagnostic aspiration of a cystic lesion should be performed with great caution to decrease the risk of secondary bacterial infection or cyst content spillage leading to secondary echinococcosis or allergic reaction. Administration of a BMZ should also accompany this procedure. Parasitological examination of the cystic material will allow for the evaluation of protoscoleces and hook morphology. The most important distinguishing features between *E. vogeli* and *E. oligarthrus* larvae are the shape and size of the large and small rostellar hooks. If rostellar hooks are absent, a definitive diagnosis of the infecting species cannot be ascertained microscopically (D'Alessandro et al., 1979). PCR followed by genetic sequencing is currently the best technique to confirm the infecting species (Grenouillet et al., 2013; see also Chapter: Laboratory Diagnosis of *Echinococcus* spp. in Human Patients and Infected Animals by Siles-Lucas et al., 2017).

3.2.5 Differential diagnosis

In tropical regions of Central and South America, abdominal echinococcosis is often misdiagnosed as malignant hepatic neoplasms, cholangiocarcinoma, abscesses, liver cirrhosis, endometrial neoplasia, gastric cancer or rib chondrosarcoma. The differential diagnosis, for pulmonary echinococcosis, often includes abscesses and lung cancer (D'Alessandro, 1979; Siqueira et al., 2013). CT is considered the best imaging method for differentiating between NE and neoplastic diseases.

3.3 Clinical management and follow-up

Since there are no prospective studies evaluating treatment of NE patients, case management should be based on the perceived benefits and risks of each treatment modality (D'Alessandro and Rausch, 2008; D'Alessandro, 2010; Siqueira et al., 2013). Treatment, for polycystic NE, has been based on recommendations given for CE (Brunetti et al., 2010). Unfortunately, patients often present in an advanced stage of disease, which may make surgery not a viable option. In these cases, other treatment and other options need to be implemented, e.g., long-term antiinfective drug treatment methods should be considered, including long-term therapy with a BMZ. The usefulness of percutaneous techniques has not been assessed. However, the polycystic nature of the condition would appear to make treatment with percutaneous methods challenging. Additional studies are needed to better determine whether a patient with polycystic NE should receive a BMZ alone or be treated with a percutaneous treatment or surgical technique (D'Alessandro and Rausch, 2008; Siqueira et al., 2013).

It has been suggested that a treatment strategy, similar to how AE is managed, may be a better fit for pathological features of polycystic NE. Siqueira et al. (2013) adapted the AE classification scheme (PNM classification) (Kern et al., 2006) for cases of polycystic NE. This classification (see Table 4) considers the appearance of the cyst in the liver, the invasion of neighbouring organs and metastases to other organ systems. While useful for hepatic cysts, this classification may not be appropriate for patients with lesions exclusively in the peritoneal cavity. These cysts would likely be staged as PNM IIIb or IV, which may not correspond with the actual disease severity (Fig. 42). Therefore an updated classification taking into account cysts located in the peritoneal or retroperitoneal spaces has been proposed (Siqueira et al., 2010, 2013). Video-laparoscopy may be a useful method to help classify patients according to the modified scoring system as well as to better identify isolated liver or peritoneal cavity lesions and evaluate whether or not the cyst(s) can be managed surgically.

Many of the surgical recommendations, for the management of AE, should be applicable to polycystic NE. However, additional studies should be conducted at NE referral centres in endemic areas. The biggest treatment obstacles are being able to diagnose NE early in the disease process, thus allowing for a curative surgical approach. Liver transplantation may be indicated for patients with PNM stage IIIa, IIIb or IV cysts suffering from portal hypertension and signs of severe liver failure, although there are currently no reports on liver

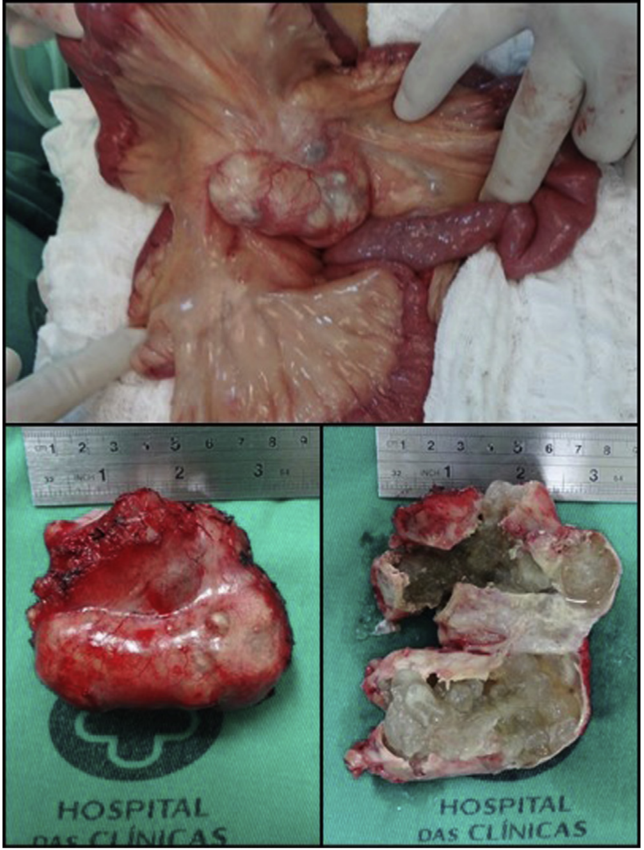


Figure 42 Polycystic neotropical echinococcosis. View of the abdominal situs during operation and specimens of gastrectomy, showing multiple metacestode nodes throughout the peritoneal cavity. Computed tomography scan from the operated patient is shown in Fig. 40. *Courtesy by Nilton Siqueira.*

transplantation in polycystic NE. In patients with cysts in the peritoneal cavity resection can be problematic, particularly in situations involving hundreds of cysts that compromise vital structures such as the vascular pedicles. Patients with PNM stage IIIa, IIIb and IV cysts should first be treated with a BMZ, and reevaluated later for a possible surgical intervention. There has been some success in treating patients with cysts larger than 50 mm in diameter, they can be treated using percutaneous techniques (Siqueira, personal communication). However, percutaneous techniques require further study in patients with polycystic NE, to determine morbidity rates and to compare

outcomes with patients treated with long-term BMZ therapy. As for CE and AE, long-term follow-up of NE patients is essential to assess treatment outcomes and to evaluate therapeutic strategies for different cyst stages and locations. Patient follow-up relies on imaging techniques and should follow the same rules as those presented for AE. At this time, serology is not considered to be an adequate tool, in itself, to monitor patients' posttreatment.

3.4 Outcome and prognosis

Based on published data, polycystic NE has a 5.2% mortality rate in patients treated surgically (operative/postoperative). When considering all cases of polycystic NE that are treated either surgically or medically, mortality rises to 15.5% (Siqueira et al., 2013). These high mortality rates indicate that polycystic NE is as dangerous as AE when it is diagnosed in its clinical course (Wilson et al., 1992). Table 9 provides outcome of 58 prospectively studied polycystic NE patients. Of the nine patients who died, seven had PNM stage IIIb cysts, one had a stage IV cyst, and one had a stage I cyst. All deaths resulted from portal hypertension or hypertension due to extrinsic compression by the cyst. Another study reported an overall mortality of 29% among 78 polycystic NE patients with complete medical histories (D'Alessandro, 2010). While surgical treatment appears to produce better results than medical treatment alone, pre- and postoperative ABZ combined with surgery is considered the most effective treatment regimen when cyst resection is feasible (Siqueira et al., 2007, 2013).

Table 9 Treatment and evolution of the polycystic neotropical echinococcosis

Treatment and evolution	Prospective study[a] No of cases (%)
Total	**58 (100)**
Surgical treatment	25 (43.1)
Percutaneous treatment	5 (8.6)
Albendazole treatment only	28 (48.3)
Operative mortality	3 (5.2)
Mortality due the disease evolution	6 (10.3)
Total mortality	9 (15.5)
Total cure	19 (32.8)

[a]Adapted from Siqueira, N.G., de Siqueira C.M., Rodrigues-Silva, R., Soares, M. do C.P., Póvoa, M.M., 2013. Polycystic echinococcosis in the state of Acre, Brazil: contribution to patient diagnosis, treatment and prognosis. Mem. Inst. Oswaldo Cruz 108, 533–540.

3.5 Burden of neotropical echinococcosis

Polycystic and unicystic NE are considered rare diseases and no studies have been conducted to evaluate their monetary and nonmonetary burden in any of the 15 Central and South America countries where NE in known to occur. Individuals living in rural settlements in or near tropical forests where known wildlife hosts for *E. vogeli* and *E. oligarthrus* reside are at risk of infection. However, individuals living in peri-urban areas on the periphery of tropical forests may be also at risk. In addition, human activities that encroach on wildlife habitats (e.g., ecotourism, sport hunting and/or poaching, land development) may also put additional groups of people at risk. Epidemiological studies are needed to better elaborate risk factors for infection and disease burden on impacted communities and populations.

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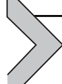

ANNEX 1

Details for percutaneous procedures used to treat uncomplicated liver cystic echinococcosis.

General preparation

All patients are receiving 10 mg/kg/day ABZ for 1 day prior to and 4 weeks following the procedure. Patients are fasted for at least 6 h before the procedure. Complete blood counts (CBC), partial prothrombin time (PTT) and international normalized ratios (INR) of the patients is required before the procedure. All procedures are performed in an operating theatre or in an interventional radiology room with ultrasonography and fluoroscopic guidance. General anaesthesia is provided during the procedures.

Puncture-aspiration-injection-reaspiration

The first puncture is performed through the liver parenchyma under ultrasonography guidance and by using 18—19 Gauge needle after the safe entry tract has been decided. The safe entry tract includes an angle of puncture so that normal liver parenchyma is present between the puncture point and the

surface of the cyst to avoid leakage of cystic fluid (prevention of anaphylactic reactions) and spillage of protoscoleces (prevention of recurrence). Direct puncture of a cyst close to the abdominal wall must be avoided. It also includes absence of vascular structures from the entry site to the cyst. As soon as the cyst is punctured the cystic content is aspirated and hypertonic saline solution (20—30%) or alcohol (95%) is injected into the cavity. It is recommended to wait for approximately 10 min before complete reaspiration of the cystic content.

Standard catheterization technique

In this technique the cyst is punctured under ultrasonography guidance with a Seldinger needle and most of the fluid content of the cavity is aspirated. Absence of communication between cyst and biliary system must be checked by injection of contrast media into the cavity. After exclusion of a fistula, hypertonic saline (30%) is injected into the cavity. Before injection of hypertonic saline the volume of the cyst fluid is measured after aspiration and contrast administration (cavity volume). After 10 min, a 0.035-inch Amplatz (Boston Scientific, USA) guide-wire is advanced under the fluoroscopic guidance before the placement of a small 6—*8 French* calibre pigtail catheter using the Seldinger technique. After the irrigation with hypertonic saline, the catheter is fixed to the skin and left for gravity drainage. When the daily drainage of the cavity is less than 10 mL, a cystogram under fluoroscopic guidance is obtained for the assessment of possible cysto-biliary communications. After ruling out the possibility of the leakage, absolute alcohol (95% alcohol in an amount 30—50% of the aspirated cavity volume) is injected into the cavity. After waiting for 5—10 min, all cystic content, including alcohol, is re-aspirated before the catheter was taken out. If the daily drainage is more than 10 mL, the catheter is kept in place until the daily drainage ceased, and then, sclerosis is performed using 95% alcohol under fluoroscopic guidance. This is followed by the withdrawal of the catheter.

Modified catheterization technique (MoCaT)

The major goal of MoCaT is the removal of the cystic content, including both liquid and solid parts (e.g., parasitic membranes, daughter cysts). After the initial puncture by an 18 or 19 Gauge needle under ultrasonographic guidance, contrast media is injected into the cavity and a 0.035-inch Amplatz guide-wire is immediately advanced under fluoroscopy guidance. Hereby, some of the daughter cysts are mechanically destroyed. Tract dilation up to 14French, up to the border of the cyst wall, is achieved under

fluoroscopy. A 14French pigtail catheter is advanced over the guide-wire, and placed within the cavity. Isotonic saline is injected into the cavity using a 10–20 mL syringe and, the same volume as injected, is aspirated. By the action of injection and aspiration some pieces of membranes are also aspirated besides fluid contents via a 14French catheter. This action is repeated more than 100 times to finally evacuate all the content. Otherwise, further sessions are employed to achieve this aim. This type of irrigation is as aggressive as effective (Akhan et al., 2007b). If the daily drainage becomes less than 10 mL, sclerosis of the cavity is performed by injecting 95% alcohol under fluoroscopic guidance, and then, the catheter will be removed. If the daily drainage exceeds 200 mL, the patient is referred to the department of gastroenterology/digestive endoscopy for papillotomy to reduce the biliary pressure and to accelerate the closure of the fistula between the biliary tree and the cavity.

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