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Review Article

Nothing is perfect! Trouble-shooting in immunological and molecular studies on cestode infections*

AKIRA ITO

Department of Parasitology, Asahikawa Medical University, Asahikawa 078-8510, Japan

Corresponding author: Tel: +81 166 68 2686. Fax: +81 166 68 2429.
E-mail: akiraito@asahikawa-med.ac.jp

SUMMARY

This unique review focuses on ways to approach and overcome some of the more common issues encountered while studying cestode zoonoses. The information presented here is based on the author's own experiences with immunological and molecular approaches for the detection of these parasites. There are many incongruities between immunological and molecular studies due to biased work. Nothing is perfect. Indirect approaches using either immunological, or even molecular tools, are limited without confirmation from direct evidence of infection. The dilemma of whether developing countries should develop their own diagnostic tests or rely on commercially available kits is also discussed.

Key word: taeniases, cysticercoses, echinococcoses, diagnosis

INTRODUCTION

Zoonotic cestodiases, including echinococcoses caused by several species of the genus *Echinococcus*, cysticercosis caused by *Taenia solium*, and taeniases caused by *T. solium* and *Taenia saginata* are globally distributed. However, these conditions are considered neglected due to the lack of tools for their detection and because they are given a low priority in most countries (Ito *et al*. 2003a; Budke *et al*. 2006, 2009; Craig *et al*. 2007). *Echinococcus* spp. requires herbivorous or omnivorous mammals as intermediate hosts and carnivorous mammals as definitive hosts. By contrast, *T. solium* and *T. saginata* require omnivorous or herbivorous mammals, mainly swine and cattle, respectively, as intermediate hosts, and humans as definitive hosts (human taeniases). Recently, a third human taeniasis, caused by *Taenia asiatica*, was reported from Asia (Fan, 1988; Fan *et al*. 1990; Eom and Rim, 1993; Simanjuntak *et al*. 1997; Ito *et al*. 2003b; Eom, 2006; Flisser *et al*. 2011). While *T. solium* cysticercosis can affect humans, other *Taenia* spp., such as *T. saginata* and *T. asiatica*, cause cysticercosis solely in livestock (Ito, 1992 vs Ito *et al*. 2003b). Furthermore, cysticercoses in domestic animals are caused by numerous other non-human *Taenia* spp. such as *T. hydatigena* (Euzeby, 1974). Complicated and various life cycles and differences in pathogenicity in humans and domestic animals also lead to these conditions being considered neglected (Budke *et al*.

2009). How to best evaluate human cysticercosis is still a complicated question and more data are required to better assess risk factors associated with disease transmission.

In order to move towards improved treatment and control or eradication of cestode zoonoses, we need to establish highly reliable indirect tools for detection of parasite carriers. Since *Echinococcus* spp. requires mainly wild animals for the completion of its life cycle, with the exception of *Echinococcus granulosus* (= *E. granulosus* sensu stricto, Nakao *et al*. 2013a) which predominately has a domestic dog-livestock cycle, establishing control interventions can be quite difficult. Due to the severity of disease caused by some species of *Echinococcus*, such as *E. multilocularis*, which causes alveolar echinococcosis (AE) and can resemble hepatic cancer, some endemic countries, such as China, have started to give echinococcosis a higher priority.

By contrast, cysticercosis, due to *T. solium*, is commonly neglected because asymptomatic taeniasis carriers are not officially detected and do not receive treatment. In addition, cysticercosis is mainly endemic in poor villages where people eat pork without meat inspection, with these populations having little political will (Ito *et al*. 2003c). It should be possible to eradicate human cysticercosis since transmission is based on hygiene and food preparation practices (Schantz *et al*. 1993). Side effects may occur when cases of human cysticercosis are treated with praziquantel (PZQ), however, the severity and extent of these side effects is still largely unknown due to lack of data (Pawlowski, 2006; Takayanagui *et al*. 2011; Jung-Cook, 2012; Baird *et al*. 2013; Ito *et al*. 2013).

In this review article, I will summarize the importance of the application of modern diagnostic tools for detection of humans and animals infected with these zoonotic cestodes. I will also discuss how to evaluate the tools themselves, and address the importance of real-time detection of taeniasis carriers. My purpose in writing this unique review is to stress the importance in doing less biased work through some cautionary tales from my own work (Ito, 1992), remembering that nothing is ever perfect.

INDIRECT VERSUS DIRECT EVIDENCE OF INFECTION

Diagnostic imaging, antibody responses, and clinical background are very important to help clinicians come to a definitive diagnosis of cysticercosis or echinococcosis before

treatment. However, advanced imaging (for example, ultrasound, computed tomography, and magnetic resonance imaging) for cysticercosis and echinococcoses are not always readily available and clinicians require specialized training to adequately interpret available images. Neurocysticercosis (NCC) cases are often asymptomatic in endemic areas. However, cases of subcutaneous cysticercosis (SCC) often have visible or palpable lesions, with reports of SCC common in Asia (Ito *et al*. 2003d; Kobayashi *et al*. 2013). The only truly pathognomonic advanced imaging feature for cysticercosis is visualization of an invaginated scolex in a cyst wall (Ito *et al*. 2006; Nash and Garcia, 2011; Del Brutto, 2012). In AE cases, imaging may look similar to other space-occupying diseases, including hepatic cancers and other hepatic conditions, including fascioliasis or amoebiasis (Eckert *et al*. 2001; Bresson-Hadni *et al*. 2006, 2011; Yang *et al*. 2007; Brunetti *et al*. 2010; Li *et al*. 2010).

More than one decade ago, major newspapers, in Japan, reported a single AE case on the main island of Honshu. These reports stated that this killer parasite had invaded the main island from the endemic island of Hokkaido. However, this information was based solely on serology using crude antigen, which was not evaluated for cross reactions with more common parasites such as *Fasciola* spp. If the researchers had checked antibody responses using a panel of other parasitic infections, they would have determined that there was no real evidence to support a diagnosis of AE (Ito *et al*. 2002a, 2003c). At that time, I personally believed that there was no chance that *E. multilocularis* would become established on the main island of Honshu, even though there were several confirmed reports of accidental infections in pigs (Kimura *et al*. 2010), horses (Kaji *et al*. 1993; Goto *et al*. 2010; Ueno *et al*. 2012) and dogs (Yamamoto *et al*. 2006) imported from the endemic island of Hokkaido. However, after a tsunami hit Japan and atomic power stations' explosion in Fukushima emerged in March of 2011, I changed my mind. I now urge caution due to the escape of numerous livestock during post-tsunami flooding as well as food sources for livestock, on the main island, being provided from Hokkaido. As shown in Konyaev *et al*. (2013), numerous Galagos (or bush babies) in the Moscow Zoo died of AE in 2010 and 2011, due to contaminated food and mulch being brought in from an endemic area. In cystic echinococcosis (CE) cases, diagnostic imaging findings are highly variable during the different developmental stages of the cysts (Eckert *et al*. 2001; Brunetti *et al*. 2010). Therefore, clinicians often require

additional information to confirm a diagnosis, including serology and a working knowledge of the epidemiology and clinical manifestations associated with this condition.

General problems in serology

There are many review articles reporting serological studies on cestode zoonoses (Gottstein, 1992; Craig *et al*. 1996; Siles-Lukas and Gottstein, 2001; Ito 2002; Ito and Craig, 2003; Ito *et al*. 2006, 2007; Schantz, 2006; Deckers and Dorny, 2010; Nash and Garcia, 2011; Del Brutto, 2012; Bames *et al*. 2012). Serodiagnostic tools have been greatly improved based on new advanced knowledge and technology in immunology. For example, the technology originally used for the indirect haemagglutination (IHA) test has now been applied to a nano-magnetic particle agglutination test (Handali *et al*. 2010). Newly available tools have another benefit in that they use recombinant antigens or synthetic peptides which can increase test specificity. However, these new tools are expensive and often under patent, which restricts their use in poor developing countries where neglected tropical diseases (NTDs) such as echinococcosis and cysticercosis are prevalent (Handali *et al*. 2010; Lee *et al*. 2011).

When we used IHA two to three decades ago, our ability to purify and apply diagnostic antigens was still in the early stages. IHA or enzyme-linked immunosorbent assays (ELISA) using hydatid cyst fluid (HCF) from *E. granulosus* s.s. still lack specificity for the detection of CE, but may be "better or much better than nothing in mass screening where CE is highly endemic" (Mamuti *et al*. 2002; Mohammadzadeh *et al*. 2012). In contrast, HCF is of little use for screening or detection of AE cases (Yu *et al*. 2008). There is a report that describes specific antibody responses in horses naturally infected with *E. multilocularis* (Ueno *et al*. 2012). However, the quality of the Western blot (WB) results from the one AE sample used as a positive control was poor compared to the results by Tappe *et al*. (2008). The findings strongly suggest that the quality of the commercially available WB membrane was also very poor or expired. It is my belief that a positive result might have been obtained regardless of the infection status of the horse. Nonetheless, these serum samples appear to be useful or informative for further studies.

Nowadays, we have improved skills for preparing highly purified antigens using new

biotechnological tools for the production of recombinant and synthetic proteins. There are those with the opinion that personnel in endemic areas should buy highly reliable test kits for mass screening or identification of individual patients. However, in the past, when such kits were widely available commercially, the quality of the kits often was very bad with poor quality control. These unreliable diagnostic kits can still be found for sale today. One solution is that people in endemic areas or countries use their own skills and knowledge to set up reference centers for the detection of infected humans and animals. Experts in developed countries can be called upon to lend their expertise to help develop appropriate diagnostic strategies in developing countries. There are many good diagnostic materials which are readily available to conduct serology in endemic areas. Therefore, we need to consider simple but reasonably reliable tools which are easily introduced into these areas (Sako *et al*. 2013).

ELISA versus *WB*

It is widely believed that ELISA is highly useful for serological screening, but WB is only helpful for confirmative serology. However, is this really correct? It might be correct only when we use crude antigens, including cyst fluid from *T. solium* or other related species, such as *T. hydatigena* or *T. crassiceps,* or HCF or crude antigens from *Echinococcus* spp. These cyst fluids are, nonetheless, much better than crude antigens extracted from the whole intact parasite, due to lesser amounts of non-specific components. When we used crude antigens for ELISA, it was impossible for us to differentiate specific antigen-antibody responses. These specific responses are more useful for diagnosis than non-specific responses or antigen-antibody responses, which are read based on a change in color of the ELISA solution and do not correlate as well with diagnosis. However, if we use the same antigens for WB, we can visualize the specific antigen-antibody responses as unique band(s) among a myriad of other bands not helpful in diagnosis. Therefore, when we use such crude antigens with both specific and nonspecific components, it is essential to be able to recognize and confirm the specific band(s) that are diagnostically meaningful. This might not, however, always be straight forward since it is possible to misidentify multiple components with similar molecular weights as a single band, through insufficient running time on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, if one

dimensional SDS-PAGE for routine WB is not sufficient to differentiate two or more components with the same or very similar molecular weights, two dimensional SDS-PAGE must be employed.

When we used highly purified antigen(s) which showed specific responses, there was virtually no difference between ELISA and WB (Ito *et al*. 1998a, 1999a, 2007; Sako *et al*. 2000, 2002; Müller *et al*. 2007). Dot-ELISA on nitrocellulose membrane may be sufficient for detection of specific antibody responses onto the purified antigen and this should be the same for the development of rapid imunochromatography (ICT) tests (Sako *et al*. 2011). Therefore, identification of specific components for diagnosis, purification of these components, and production of these components as recombinant proteins or synthetic peptides is suggested. A more important objective may be to keep or establish serum banks of confirmed human patients or animals, since it is difficult to keep a sufficient number of confirmed serum samples, especially representing different stage of these diseases. The development and maintenance of these serum banks should be facilitated by the World Health Organization (WHO) and/or the Food and Agriculture Organization of the United Nations (FAO).

Is Em18 perfect for detection of active AE?

As shown by Sako *et al*. (2002), Em18, ezrin-radixin-moesin (ERM)-like protein (ELP), encoded by the gene *elp* (Brehm *et al*. 1999), is a degradation product through cysteine proteases. Four components of ELP (EMII/3 (Gottstein *et al*. 1988), EM10 (Frosch *et al*. 1991), EM4 (Hemmings and McManus, 1991) and Em18 (Ito *et al*. 1993a)) have been reported by four different groups. Em18 corresponds to a region with very limited homology between the host and parasite ERM factors, which indicates that serology using Em18 as an antigen might lead to more specific responses when compared with full-length ELP (Ito *et al*. 2007). This strongly suggests that degradation products may be detected, not from the early stage, but rather from the later stage of AE. If this is true, it might suggest that detection of antibody responses to recombinant Em18 (RecEm18) is useful for AE cases with active and advanced lesions (Ito *et al*. 1995; Tappe *et al*. 2008, 2009, 2010). According to Li *et al*. (2010b), 67% of AE1 cases were positive to RecEm18, whereas 80%, 90% and 97% of AE2, AE3 and AEf cases, respectively were positive to RecEm18. Therefore, the question arises of, “How should we interpret these

findings for AE1"? Is it possible for us to look for other components for serodiagnosis of the early stage of AE? *E. multilocularis* metacestode vesicle fluid (EmVF) producing bands at 20-22 kDa (Müller *et al*. 2007) and *E. multilocularis* major vault protein (MVP) (Goto *et al*. 2013) may be alternative candidate antigens for diagnosis of early stage AE cases if they prove to be more sensitive and specific than RecEm18.

Recently, we were presented with an early stage AE case, with a hepatic lesion measuring approximately 1 cm in diameter. The patient was sero-negative by both confirmative WB using crude antigen carried out at the Hokkaido Institute of Public Health and RecEm18-WB carried out at Asahikawa Medical University (AMU). However, when tested using crude antigen-WB at AMU, I found one very strong band with a high molecular weight. It completely differs from any diagnostic components of a commercially available WB kit (Liance *et al*. 2000). Therefore, crude antigen-WB might also be useful for the serological detection of early stage of AE (Hagiwara *et al*. in prep.). However, further studies are necessary. The use of highly purified antigen can detect most, but not 100% of true cases, with very few false positives. However, when we use crude antigen, we may detect more than 100% of cases with substantial numbers of false positives (Fig. 1). Approximately one decade ago, serology applied in Hokkaido, Japan using crude antigen-ELISA, showed that approximately 99% of cases positive by crude antigen-ELISA were false positives. Unfortunately, this serological test sometimes also failed in the detection of true AE cases which were easily confirmed by RecEm18-WB (Ito *et al*. 2003b, c). Using crude antigens, we estimate that we obtain a very large number of false positives and, possibly more importantly, false negatives. However, it is not sure if false negatives are quite a few or not (Ito *et al*. 2002a, 2003c; Aoki *et al*. 2006). In contrast, Em18-serology has proven to be much better for the detection of AE cases, with almost no false positive cases in Japan (Ito *et al*. 1993a, 2003c, Aoki *et al*. 2006), China (Ito *et al*. 1993b), USA (Ito *et al*. 1995), Poland (Ito *et al*. 1998a), France (Bart *et al*. 2006) and Germany (Tappe *et al*. 2008, 2009, 2010). There are a few cases of early stage AE that have shown no antibody response to Em18 (Hagiwara *et al*. in prep.). However, I expect these cases will become positive over time. There is no way to determine the number of false negative AE cases other than to utilize different antigenic components or tools on stocked serum samples. Therefore, we must decide on what is our true objective: Detection of 96-97% of AE cases with no false

positives or detection of 98% of AE cases with high numbers of false positives. An alternative idea is to utilize fine needle aspiration for histopathological and molecular confirmation of all stages of AE, since the risk of anaphylaxis is believed to be low for AE cases (Kern *et al*. 1995; Kawakami *et al*. 2013).

There have been few reports stating that RecEm18 does not detect 100% of active AE cases, but that other commercially available WB kit (Liance *et al*. 2000) could detect 100% of active AE cases (Furuya *et al*. 2004; Yamano *et al*. 2005). However, these reports did not include other infectious disease samples for evaluation of the test's specificity (Fig. 1). Furthermore, the authors did not use RecEm18, but instead used crude antigens for identification of Em18-WB. It is necessary to run the SDS-PAGE for a sufficient amount of time to have adequate separation of the various components (Ito *et al*. 1993, 1995, 1997, 2002a, b; Liance *et al*. 2000; Tappe *et al*. 2008) and use some specific markers such as monoclonal or polyclonal antibodies toEm16 or Em18 or any other diagnostic components (Ito *et al*. 1993, 1995, 1998a; Jiang *et al*. 2001).

In South America, three *Echinococcus* spp., *E. granulosus* sensu stricto, *E. canadensis* and *E. vogeli* are distributed. As the genes of Em18 and AgB are shared among *Echinococcus* spp. (Nirmalan and Craig, 1997; Nakao *et al*. 2009) and the expression of these genes are expected to be variable among the different pathological feature of echinococcoses (Wen and Craig, 1994), we may expect that antibody responses in *E. vogeli* infections (polycystic echinococcosis, PE) may be somewhere between that of AE and CE (Knapp *et al*. 2009; Ito *et al*. 2011a).

How rapidly do antibody responses to RecEm18 decline after curative surgery?

Most recent serological follow-up studies of cured post-surgical hepatic AE cases in Japan showed unexpected antibody responses (Fig. 2). For example, we were asked to follow-up one AE cases for 6 months after surgery. After 6 months, antibody responses to RecEm18 were already negative (data not shown). RecEm18 has been known to be a good marker to follow-up the progression of AE especially in resected AE cases (Xiao *et al*. 2003; Bart *et al*. 2007; Ishikawa *et al*. 2009; Tappe *et al*. 2009, 2010; Bresson-Hadni *et al*. 2011). However, just recently it was shown that antibody responses start to decline within only a few days post-surgery (Fig. 2-a, -c) (Akabane *et al*. 2012). We have had several AE cases show similar drastic declines in antibody

responses within one week of curative surgery, with all becoming negative within 6 months (Fig. 2-b, -d) (Akabane *et al*. in prep.). This rapid decline in antibody response may be true not only in AE cases, but also for other helminthic diseases or other conditions requiring hepatic surgery. If this is confirmed, there might be some unknown mechanism for inactivation of antibody responses after hepatectomy. Otherwise, we will need to re-evaluate the immune memory itself using purified antigen(s). At present, AE is the only parasitic disease where surgical resection of the entire lesion is recommended as the first therapeutic choice. Very active homeostatic responses in patients after surgery, anergy, or some unknown mechanism to induce a rapid drop in antibody titers after surgery may exist and need to be evaluated further.

Comparative studies using different tools including commercially available kits

There are many reports that compare the specificity and sensitivity of diagnostic tools. Studies where the test developers apply the various diagnostic tools are optimal in order to reduce bias (Ito *et al*. 2002a), unless the test(s) being evaluated are commercially available (Ito *et al*. 1998b; Tappe *et al*. 2008; Carod *et al*. 2012). Comparative studies for serological tools that are not commercialized should be carried out by the two or more groups that have been involved in the establishment of the tools (Dorny *et al*. 2004). Usually, such joint work should be carried out under blind or double blind conditions (Ito *et al*. 1993, 1995, 2002b). Otherwise, researchers who are interested in using or evaluating another group's diagnostic tool should attempt to collaborate with the test's developer (Li *et al*. 2003; Bart *et al*. 2007). In all cases when serology is used, it is important to consider ancillary findings (for example, diagnostic imaging and clinical manifestations) to support the diagnosis since no test is 100% reliable (Tappe *et al*. 2008, 2009).

Detection of specific antibodies versus *detection of circulating antigens*

Most serological approaches are based on detection of specific antibody responses. However, an alternative is to detect circulating antigens. The former cannot differentiate current and previous infections due to immunological memory after cure. However, there are no detailed follow-up studies on how long antibody responses remain after surgical resection in cysticercosis or echinococcosis cases when using purified antigens.

Usually, clinicians try to follow the disease's progression via imaging and antibody responses 6 months to one year after treatment. As an example, we were asked to check antibody responses just prior to and one year after the surgical treatment of one NCC case with a solitary lesion. The patient was seropositive before surgery, but completely seronegative one year later (Ito *et al*. 1999b). The original serology applied at the hospital was not sensitive and, therefore, the patient was believed to have a malignant brain tumor. The lesion was surgically resected and later confirmed to be NCC. A seronegative result one year post-surgery was not able to provide information on how long the antibody response remained (Deckers and Dorny, 2010). In order to determine length of antibody response, follow-up studies are required for both surgically treated and chemotherapeutically managed cases (Kobayashi *et al*. 2013).

In the past, clinicians in Asia used to perform surgery for lung paragonimiasis which, in Japan, was often misdiagnosed as lung tuberculosis (TB). However, after serology for paragonimiasis was established and the drug, Bithionol, was commercially produced, clinicians preferred chemotherapeutic therapy to surgery. Currently, we have no serum samples from surgically treated paragonimiasis cases. If it is possible to acquire samples from such cases and perform weekly or monthly post-surgical follow-up, we should be able to determine if there is also a rapid decline in antibody titers with this parasitic disease. Another interesting research topic could be how rapidly antibody responses in NCC cases with surgery become negative post-surgery (Deckers and Dorny, 2010).

Serology to detect circulating antigens is expected to show evidence of an ongoing infection. However, the specificity of these tests is still not entirely known. Almost all antigen tests have been designed to detect certain components of metacestodes of *T. saginata*, but not of *T. solium*. This means that the current antigen-based tools for *T. solium* cysticercosis are based on cross reactions. The question is, "Why has no one tried to produce specific antibodies to specific components of the metacestodes of *T. solium*?". "*T. saginata*" based tests might be useful for detection of human cysticercosis, since *T. solium* is essentially the only species which can infect humans other than very rare cysticercosis cases caused by non-human *Taenia* spp. common in wild animals (Euzeby, 1974). However, if this tool is used for the detection of pigs infected with *T. solium*, there is no doubt that positive results would occur not only for pigs infected with *T. solium*, but also for pigs infected with other taeniid cestodes, including *T.*

hydatigena. A joint project to evaluate antibody-ELISA and antigen-ELISA under blind testing resulted in reasonably reliable results for the antibody-ELISA, with all pigs harboring 16 or more cysticerci at necropsy 30 days after egg inoculation confirmed antibody positive (Sato *et al*. 2003). In contrast, the sera from one uninfected pig became antigen-ELISA positive based on the utilized cut-off.

Which is better to use experimentally infected or naturally infected animal sera?

Experimental infections to establish specific serodiagnosis are widely used. The candidate diagnostic antigens are then applied for the detection of specific antibody responses in patients or animals infected with the parasite species of interest. As *T. solium* endemic areas tend to be located in poor regions of developing countries, people and animals are often infected with multiple pathogens, including several other helminthes. Therefore, serum samples from endemic areas are very useful for the establishment of better diagnostic tests with higher specificity (Ito *et al*. 1999a). It is, for this reason, that we often discuss how to establish negative controls. Ideally, negative controls from endemic areas are better than negative controls from non-endemic countries. However, if the antigens applied are specific enough, there may be no difference between negative controls from endemic and non-endemic areas (Nkouawa *et al*. 2011; Mohammadzadeh *et al*. 2012).

Pigs in developing countries are commonly infected with *T. hydatigena* and infection with this parasite appears to be more common than infection with *T. solium* in Asia (China, Thailand and Indonesia). There is a report that *T. hydatigena* is not common in Africa (Dorny *et al.* 2004). However, no one knows if it is always and everywhere true. Although we had no data on *T. hydatigena* from pigs that were confirmed to be co-infected with *T. solium*, we applied our serological test, developed to detect human cysticercosis, and found pigs naturally infected and confirmed positive for *T. solium* infection in Indonesia (Papua), China, and Mexico (Ito *et al*. 1999a). Such results from pigs confirmed to have been naturally infected with *T. solium* were ideal. Therefore, we should use serum samples from pigs grown in endemic areas for evaluation of serology.

There has been a push to use an WB kit using glycoproteins (GP-WB) established for detection of human cysticercosis (Tsang *et al*. 1989) for swine cysticercosis (Garcia *et al*. 2003; DeGiorgio *et al*. 2005; Mwape *et al*. 2013). However, the GP-WB may only

be specific in humans who are exclusively infected with *T. solium*. This means that there is no real control for evaluation of these GPs in animals which can be infected with other taeniid cestodes. There are several reports discussing "transient antibodies" detected by the GP-WB in swine cysticercosis (Garcia *et al*. 2003; DeGiorgio *et al*. 2005; Mwape *et al*. 2013). Therefore, it is suspected that some of the GPs are shared with other non-human *Taenia* species (Lightowlers, 2013).

Evaluation of serological results

Use of advanced active cases of echinococcosis or cysticercosis to evaluate serodiagnostic tools should increase test sensitivity. Therefore, serum samples with good clinical background information are essential in the evaluation of serological studies. In general, advanced cases are much easier to detect by any tool. If we use purified antigens, the specificity becomes very high, but simultaneously the sensitivity tends to be lower. In contrast, if we want a test that is 100% sensitive, we usually sacrifice specificity. For a disease such as AE, it is essential to use a panel of serum samples from other diseases such as hepatic cancers, cystic echinococcosis, cysticercosis, fascioliasis, toxocariasis, amoebiasis, etc. The most important prerequisite for such panels is that the panel sera have been confirmed antibody positive to the homologous parasite or pathogen's antigen. However, even with such careful analysis, we may find shared epitopes among cestode or platyhelminthes, especially in terms of hydrophobic ligand binding proteins including Antigen B family (Iopposo *et al*. 1996; Ito, 2002; Mamuti *et al*. 2007; Jiang L *et al*. 2012; Mohammadzadeh *et al*. 2012; Santivañez *et al*. 2012; Obal *et al*. 2012).

Serology for detection of taeniasis

There are serological tools available for the detection of taeniasis carriers due to *T. solium*. However, to my knowledge, there is no scientifically sound work on how species specific these tests are. Some group used no or few samples from *T. saginata* or *T. asiatica*, but stressed that their newly developed serological test was 100% specific for *T. solium*. If sera from people infected with other *Taenia* species were seronegative to the homologous antigens, it resulted in a negative test. It is, therefore, difficult to evaluate such work without blinded tests with greater numbers of samples. Nonetheless,

even if the test cannot differentiate species, it may still be useful for the detection of taeniasis carriers. It should also be noted that such a serological tool for the detection of taeniasis carriers cannot differentiate ongoing infection from past infection due to immunological memory. Therefore, confirmation is needed on how long the antibody responses remain after treatment. This problem pushes us to develop better tools for molecular identification. Better parasite identification is also needed to assess anthelminthic drug therapy. Salim *et al*. (2009) used commercially available serological tools in Papua, Indonesia to perform serological screening of the local population. The study reported the proportion of people who were taeniasis carriers or cysticercosis patients. However, there was no direct evidence to confirm which *Taenia* species were causing infection. We require better direct evidence of these parasites in people (Wandra *et al*. 2000) pigs (Subahar *et al*. 2001; Margono *et al*. 2003) and even dogs (Ito *et al*. 2002c) in Papua (Wandra *et al*. 2013). Nonetheless, there are still better data on human cases of echinococcoses, cysticercosis, and taeniases compared with data on infections in animals.

Problems with animal surveys

Detection of animals parenterally infected with metacestodes of *Echinococcus* spp. or *Taenia* spp. is difficult due to the cost of performing diagnostic tests. There are no practical tools for the detection of animals infected with *E. granulosus* sensu lato. The same is true for other *Echinococcus* spp. infections in South America (Knapp *et al*. 2009; Santose *et al*. 2012). If echinococcoses were a more lethal in livestock and other high value animals, the animal sector would be more likely to set up sustainable screening for infections in animals.

In contrast, cattle infected with metacestodes of *T. saginata* are very rare in developed countries; with most cases the result of infected employees from developing countries contaminating farming areas by defecating in fields containing livestock (Dorny and Praet, 2007; McFadden *et al*. 2011; Yanagida *et al*. 2012; Yamasaki, 2013). If we introduce stool examination for all employees working in farming areas, it might be cheaper than introducing a new serological tool to detect infected cattle.

Copro-ELISA for detection of adult tapeworm carriers

Copro-ELISA has been useful for detection of adult worms in definitive hosts, including humans with taeniases or dogs and/or foxes infected with *Echinococcus* spp. (Allan *et al*. 1990, 1996; Deplazes *et al*. 1994, 1999; Allan and Craig, 2006). There are several groups which have been working on copro-ELISA tests. Most of them use polyclonal antibodies to capture antigens in fecal samples. These tests performed well in laboratory based studies, especially when known positives were compared with uninfected negative controls. However, in field studies in endemic areas where other parasitic infections were common, it became much more difficult to interpret test results (Raoul *et al*. 2001). Copro-ELISA appeared to work better in earlier projects,, indicating that expired capturing antibodies prepared approximately 2 decades ago may be affecting the test's outcome in more recent studies (Hartnack *et al*. 2013). In order to address the problem with specificity, some groups have applied monoclonal antibodies to capture the antigens (Nonaka *et al*. 1996; Morel *et al*. 2013). However, most of the antigenic components in faeces were not proteins, but rather glycoproteins or lipoproteins, which may result in non-specific responses. Even if such antibodies for capturing proteins, sugars, or lipids were specific, there might be blockers or inhibitors in the fecal samples. Therefore, it is not possible to know if these copro-ELISA tests are useful without direct evidence of infection (for example, eggs or tapeworms) from copro-ELISA positive humans or animals.

Molecular identification of cestode species

Molecular identification is almost 100% reliable when applied to parasites collected from human patients or infected animals. However, contamination of tools with other DNA may cause erroneous results. Therefore, repeated analyses of the same samples at different laboratories may be important for evaluation of the results (Hüttner *et al*. 2008; Knapp *et al*. 2009; Snabel *et al*. 2009). There is also the risk of contamination, especially by beginners, which may even result in unreliable sequences being inputted into GenBank.

Eggs, metacestodes and adult tapeworms are all targets for molecular identification. Even though eggs of taeniid species cannot be differentiated morphologically, they can be differentiated using molecular tools. Based on the obtained molecular data, we should treat tapeworm carriers and collect adult worms. Morphology and molecular

information from adult worms should also be compared with molecular data obtained from parasite eggs.

The merit of using coproDNA detection is that DNA of immature worms can be amplified. Therefore, coproDNA detection may be more sensitive and reliable for all stages of the worm's development in the host intestine. Polymerase chain reaction (PCR) has widely been applied for coproDNA detection (Yamasaki *et al*. 2004). However, inhibitors in the faeces may interfere with the test. Recent approaches using loop-mediated isothermal amplification (LAMP) for copro-tests, coproLAMP, are more reliable, with almost no influence from such inhibitors (Nkouawa *et al*. 2009, 2010, 2012).

Recent molecular studies of *Echinococcus* spp. have revealed that *E. granulosus* sensu lato consists of 5 independent species: *E. granulosus*, *E. equinus*, *E. ortleppi*, *E. canadensis* and *E. felidis* (Nakao *et al*. 2007, 2010, 2013a; Hüttner *et al*. 2008; Knapp *et al*. 2009). Furthermore, the hermaphroditic nature of cestodes allows them to reproduce without sexual reproduction. However, infection with multiple worms may result in outcrossing. Such data may be obtained for *Echinococcus* spp. when we compare mitochondrial (haploid) and nuclear (diploid) DNA from *Echinococcus* worms in a single definitive host co-infected with different species of *Echinococcus* or different genotypes of worms. There are also reports stressing that *E. granulosus* and *E. multilocularis* parasitize different parts of the dog intestine, while other reports state that the two species parasitize the same regions of the intestine (Thompson and Eckert, 1983; Kumalatilake *et al*. 1986; Lymbery *et al*. 1989). However, it is not known if these reports can be generalized to all dogs and other canids. There might be hybrids of, or at least introgression between, *E. granulosus* s.s. and *E. canadensis* (Barts *et al*. 2006) or other species of *E. granulosus* s.l. where these species are co-endemic. Therefore, it is possible that morphologically indistinguishable hybrid species may be found in the small intestine, but additional data are needed.

Evidence of outcrossing has been suggested from *E. multilocularis*, in Hokkaido, Japan, using microsatellite DNA (Nakao *et al*. 2003) as well as from *T. saginata* and *T. asiatica*. In these cases, although the mtDNA indicated *T. asiatica*, nuclear DNA indicated *T. saginata*, or highly variable heterozygotes of both species, and *vice versa* (Okamoto *et al*. 2010; Yamane *et al*. 2012, 2013). Experimental infection with eggs of *T.*

513 *asiatica* by Fan *et al*. (1990) resulted in *T. asiatica* metacestodes developing in the
514 viscera of pigs, but also produced metacestodes in cattle and other domestic animals.
515 This led to additional studies on this new Asian *Taenia* species (Simanjuntak *et al*. 1997;
516 Wandra *et al*. 2013; Yamane *et al*. 2013), which leads to the question of "Are there any
517 other human *Taenia* species"? In Ethiopia, cysticerci from a new *Taenia* species have
518 recently been confirmed from cattle (Hailemariam *et al*. 2013). We, therefore, are facing
519 a more complicated world in the area of taeniid cestode taxonomy (Nakao *et al*. 2013b).
520 Coevolution of cestodes and host animals are other emerging topics.
521
522 *Pathogenicity of* Echinococcus *spp. to humans*
523 Recent molecular studies have revealed that *E. granulosus* s.s. and *E. felidis* (Hüttner *et*
524 *al.* 2008), and *E. multilocularis* and *E. shiquicus* (Xiao *et al.* 2005) are sister species,
525 respectively, with the former species, of both pairs, known to be highly pathogenic to
526 humans (Nakao *et al*. 2010; Knapp *et al*. 2011). Therefore, it is hypothesized that these
527 two newer species, *E. felidis* in Africa and *E. shiquicus* in Tibet, China can also infect
528 humans. However, we should not jump to conclusions until we have concrete evidence
529 of human infections. All CE samples due to *E. granulosus* s.s. in Africa, especially
530 where *E. felidis* has been confirmed, should be re-evaluated for the possibility of *E.*
531 *felidis*.
532
533 *The importance of the real time detection of taeniasis carriers and cysticercotic pigs*
534 We have been working on taeniasis and cysticercosis in several Asian countries. Since
535 2004, the Ministry of Education, Japan, has sponsored numerous seminars to help
536 transfer technology to scientists in Asia and Africa. Parasite materials and human
537 samples from endemic areas have been analyzed at AMU after obtaining ethical
538 approval. Molecular identification of *Taenia* species using eggs, metacestodes, adult
539 worms, fecal samples, and serology have been carried out by junior colleagues from
540 endemic countries. Unfortunately, it has been very difficult to locate identified taeniasis
541 carriers for treatment because many had moved in the time between sampling and
542 diagnosis. Therefore, we have decided to establish a real-time detection system in order
543 to identify and treat carriers during a single visit as well as immediately identify pigs
544 that show evidence of being infected (Ito *et al*. 2011). This method was first employed

in Bali in 2011 (Swastika *et al*. 2012). To use the ELISA for pigs, in the field, an ELISA reader is not required since a color change indicates a positive result. Thus far, all ELISA positive pigs examined in Bali, Indonesia, were confirmed to have *T. solium* cysticerci, with or without *T. hydatigena*. When we used an ELISA-reader to check the cut-off border line samples in the laboratory, some of these samples were weak positives and some were confirmed to harbour *T. hydatigena* on necropsy (Dharmawan *et al*. in prep.). Therefore, we believe that the use of a color change ELISA may be sufficient or better for screening and identifying pigs infected with *T. solium* under field conditions (Fig. 3). However, more work is necessary. A LAMP test that can be used in the field and does not require electricity has also been developed (Nkouawa *et al*. 2012). Real-time identification of taeniasis carriers and pigs infected with *T. solium,* in endemic areas, is essential to demonstrate risk factors for human cysticercosis.

Dilemma for intervention of cysticercosis or echinococcoses in developing countries: What is the contribution of commercially available kits?

A serious problem with the application of modern diagnostic tools is that we often use such tools but do not work to obtain direct evidence of the infection itself. This oversight should be avoided. As mentioned above, serology for the detection of cysticercosis in endemic areas of Asia is not difficult even when applying simple purification tools (Sako *et al*. 2013). Therefore, we are strongly recommending keeping metacestodes from pigs (Fig. 3) and trying to purify the diagnostic antigens using a simple and inexpensive method (Sako *et al*. 2013). Commercial kits may be more useful in developed countries, especially in the USA, since the USA has many cysticercosis cases due to refugees or immigrants from endemic areas. In addition, US citizens may bring back the parasite to USA, after visiting countries where *T. solium* infections are endemic (Serpa *et al*. 2011; Sorvillo *et al*. 2011; Yanagida *et al*. 2010, 2012; Jongwietiwes *et al*. 2011).

One serious disadvantage from the use of commercially available kits is the possibility of discouraging researchers in endemic developing countries to establish tools for their own use. Another serious issue is a lack of knowledge on how to evaluate potentially erroneous data from the kits. There are many kits on the market, with many of the tests appearing to lose reliability after being commercialized. Based on this view

point, in 2004 I started to encourage personnel in Asia and Africa to understand the mechanism of antigen-antibody responses using their own samples and develop purified antigens to establish their own ELISA or IB serological tests (Ito, 2007). However, at the same time, I was encouraged to produce rapid serological kits for AE, CE and cysticercosis by the Ministry of Education, Japan (ADAMU-AE, -CE and -CC: ICST Co. Ltd., Saitama, Japan). Evaluation of commercially available kits is recommended by the WHO, and numerous other organizations.

No test is 100% reliable, but we should challenge ourselves to get better results using confirmed patients' sera. After this, we can apply the tools on suspected cases or utilize them in epidemiological surveys for confirmation of the infection itself.

CHEMOTHERAPY OF TAENIASIS IN REMOTE AND RURAL AREAS IN ASIA

In Southeast Asian countries, eggs of *Taenia* spp. may be found through stool examination for soil transmitted helminthes (STH). However, the number of samples with *Taenia* eggs will be very small compared with eggs of the major STHs. Therefore, there is usually no further analysis for the identification of *Taenia* species, since identification of *Taenia* species is time consuming and morphological identification is dependent on the adult worm being expelled. Therefore, we need molecular tools for identification of the species. As a result of the inability in adequate identification of *Taenia* species, these cestodes have been further neglected in relation to the other STHs and/or fish-borne trematodiases (FBTs). However, if the eggs of *Taenia* spp. are identified to be *T. solium*, it means that there is a risk cysticercosis to both the carrier and his or her family members and others in the community (Montresor and Palmer, 2006). The WHO has recommended mass treatment with praziquantel (PZQ) even though there can be safety issues with mass treatment. There are records of individuals dying within days of treatment as part of mass treatment campaigns against schistosomiasis and/or FBTs (Ito *et al*. 2013), but there have been no analyses of the cause of theses sudden deaths. If the areas endemic for schistosomiasis and/or other trematodiases are also endemic for *T. solium*, these sudden deaths could be due to NCC cases who succumb due to a side effect of PZQ treatment since, in these cases, PZQ can results in acute seizures or convulsions when given without a steroid (Pawlowski 2006; Wandra *et al*. 2011). Therefore, we need to reconsider the danger of PZQ for treatment

of NCC, especially during mass treatment campaigns where there could be numerous asymptomatic cases. In Asia, we need to establish a better strategy for the detection of taeniasis and cysticercosis using highly reliable immunological and molecular tools.

CONCLUSIONS

Taeniases are neglected due to the small number of cases detected via screening for STHs and the fact that *Taenia* eggs in human faeces are impossible to identify to the species level. The highly pathogenic *T. solium* should be differentiated from the two less pathogenic species (*T. saginata* and *T. asiatica*) by morphology of the tapeworm's scolex or by molecular tools, including copro tests. Serology and imaging are still necessary for evaluation of human cases of echinococcosis and cysticercosis. A more complicated situation remains in terms of identification of animal infections. There are many acute and chronic infectious diseases. Due to the small population of known patients or carriers of cestode zoonoses, these conditions will continue to be neglected until an outbreak occurs in a developed country. As the risk of infection in people is primarily from individuals living in remote areas of developing countries, we are faced with numerous challenges for controlling these neglected cestode zoonoses. We have to keep in mind that "Nothing is perfect without direct evidence of the infection". In this article, I did not discuss vaccination trials due to a lack of individual experience. However, there are numerous references that describe this topic in detail (Lightowlers, 2006, 2010a, 2010b, 2010c, 2013; Bethony *et al*. 2011; Gauci *et al*. 2013).

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(Iran, Jordan), the Americas (USA, Mexico, Brazil, Ecuador, Peru, Argentina) and Europe (UK, France, Germany, Poland, Switzerland, Italy, Slovenia, Finland, Russia) to conduct joint collaborative work for the establishment and evaluation of serological and molecular tools for diagnosis, as well as organize international workshops and symposiums (Ito *et al*. 2003c, 2011, 2013; Ito, 2007). I have enjoyed international projects with more than 250 collaborators from 45 countries.

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Figure legends

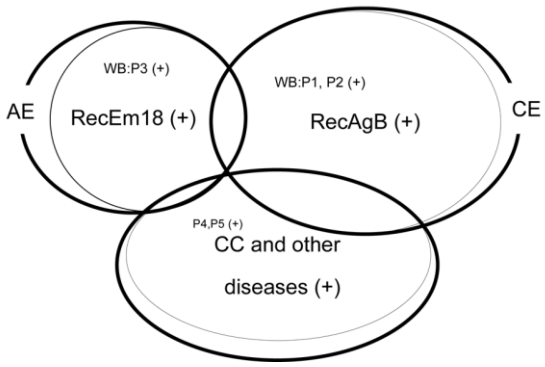

Fig. 1. Schematic figure of AE, CE and non-echinococcal cases including cysticercosis (CC) detectable by western blots (WB) using RecEm18, RecAgB (Ito *et al*. 1993b, 1995, 1997, 1998a) and commercially available crude antigen-WB P1-P5 (Liance *et al*. 2000). The majority of AE and CE cases except some early or abortive stage of AE and CE are easily detected by RecEm18 and RecAgB, respectively (Li *et al*. 2010) and by WB:P3 (WB:P3 (+) in Fig. 1) for detection of mainly Em18 (Ito *et al*. 1993b, 1995, 1997, 1998a) and WB:P1, P2 (WB:P1, P2 (+) in Fig. 1) for detection of mainly AgB (8 kDa), respectively (Ito *et al*. 1997, 1998a; Liance *et al*. 2000). WB:P4, P5 (P4, P5 (+) in Fig. 1) may include echinococcosis, either CE or AE but they are positive under blind test for CC and some other diseases and no use under a blind test or screening (Liance et al. 2000). The real confirmative WB for AE and CE are based on detection of AgB, Em16,

Em18 at least and P1, P2, P3 but not P4, P5 (Liance *et al*. 2000). Based on the banding patterns using crude antigens, we can identify AE by detection of antibody responses to Em18 or both Em18 and Em16, and CE by those responses to Em16 and AgB (Ito *et al*. 1993, 1995, 1997, 2002a, b; Furuya *et al*. 2004; Tappe *et al*. 2008). However, we cannot identify AE and CE by crude antigen-ELISA, since crude antigens have many non-specific components shared between the host and parasite. More important is that these serological tools cannot always detect early stages of AE or CE.

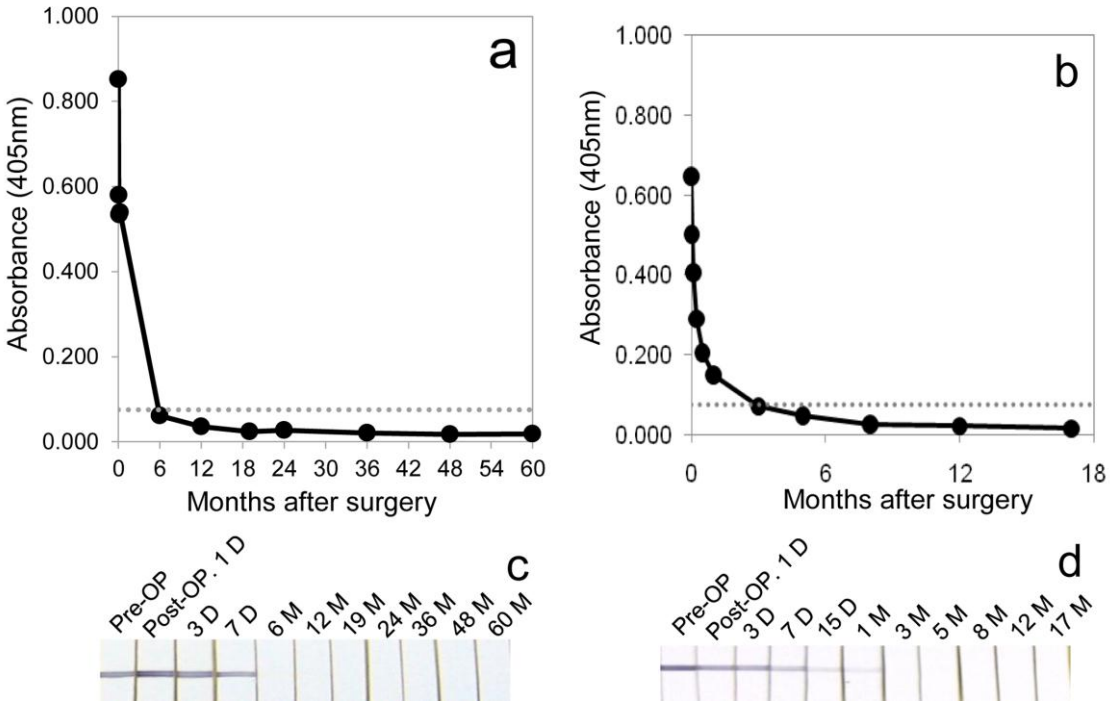

Fig. 2. The rapid declines in antibody responses in two resected hepatic AE cases (PNM stage I) (a and c: modified from Akabane *et al*. 2012; b and d: modified from Akabane *et al*. in prep.). ELISA (a and b) and WB (c and d) using RecEm18 (Sako *et al*. 2002) before surgery until 60 months (a and c) and until 17 months after surgery (b and d).

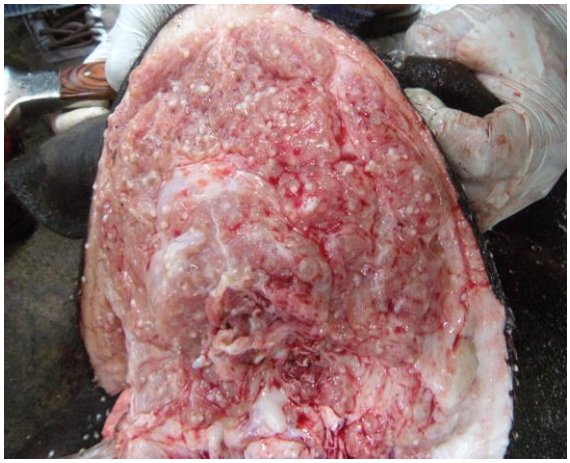

Fig. 3. A one year old pig full of cysticerci of *T. solium* in Bali, Indonesia, suspected to be infected based on ELISA in the field and judged by our naked eyes in Jan 2013. Such a pig is sufficient for local personnel to prepare huge amount of diagnostic antigens.