

# IMMUNOLOGICAL AND MOLECULAR TOOLS FOR IDENTIFICATION OF ECHINOCOCCOSIS AND FOR EPIDEMIOLOGICAL STUDIES AND THE PRESENT PROBLEMS IN JAPAN

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**Abstract.** Recent advances in immunological and molecular approaches for the better resolution of immunodiagnosis of echinococcosis, either in humans or in animals, are briefly overviewed since April 1999. National echinococcosis surveillance in Japan has been performed. However, the lack of an evaluation system to confirm echinococcosis, either alveolar or cystic forms, the echinococcosis cases reported by clinicians, especially those with no or little experience of echinococcosis have limited reliability, except for cases pathologically confirmed after surgery. This systemic problem in Japan is also discussed. The importance of establishing a scientific evaluation system to identify humans and pets infected with *Echinococcus multilocularis* in Japan is stressed.

## INTRODUCTION

Although echinococcosis, in alveolar (AE) and cystic (CE) forms, and neurocysticercosis (NCC), are not among the leading causes of parasite-induced mortality worldwide, these diseases do cause considerable morbidity in humans and economic loss in terms of health and livestock costs (Ito *et al*, 2003a). AE, CE and NCC are caused by infection with metacestode stage *Echinococcus multilocularis*, *Echinococcus granulosus*, and *Taenia solium*, respectively. Recent advances in technology for detecting these cestode infections in humans and animals are briefly overviewed (Siles-Lucas and Gottstein, 2001; Ito, 2002a; Ito and Craig, 2003; McManus *et al*, 2003; Zhang *et al*, 2003). These parasitic infections are zoonotic, techniques to detect or identify (i) patients of AE, CE, and NCC, and (ii) patients or animals infected with taeniid adult worms, are essential for control of these zoonoses. Strategies for detection of infection and control are discussed. Special attention is focused on the pre-mature stage in establishing a system for scientific evaluation of AE after sustained efforts in Japan.

## RECENT ADVANCES IN TECHNOLOGY FOR HUMAN ECHINOCOCCOSIS

There are two strategies for detection and

identification of the patients of echinococcosis: image diagnosis and serodiagnosis. It has been a matter of some debate whether image diagnosis by CT scan or ultrasonography, or serology for detection of specific antibodies, is a more useful, reliable or practical approach for detecting of hepatic AE. If image figures are typical for either CE or AE, serodiagnosis is not always needed. However, if such figures are atypical, it is important to add serological confirmation using highly specific antigens. WHO is now recommending image diagnosis as the first choice to detect hepatic abnormality, not only in highly endemic areas, but also in developed countries where AE is rare, and the use of serology with specific antigens for confirmation (Pawlowski *et al*, 2001; Ito and Craig, 2003).

### Alveolar echinococcosis (AE)

Specific antigens for detection of AE, EM10 (Frosch *et al*, 1991), EM4 (Hemming and McManus, 1991), EmII/3 (Vogel *et al*, 1988), EmII/3-10 (Muller *et al*, 1989), and Em18 (Ito *et al*, 1993) are expected to be reasonably useful. Sako *et al* (2002) revealed the biochemical and molecular correlations of all these candidate antigens: EM10 is the ezrin-radixin-moesin (ERM)-like protein of *E. multilocularis* and EM4, EmII/3 and EmII/3-10 are fragments of EM10. Em18 is a degradation product of EM10 by cysteine protease(s). It has been found that Em18 has the lowest homology with human ERM among the EM10 family, and therefore, is expected to be more sensitive in detection of AE cases (Sako *et al*, 2002).

Recent work using recombinant Em18 for differentiation of AE and CE from other parasitic diseases in blind tests has revealed that Em18 is highly sensitive and specific to AE, although minor CE with

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multiple cysts may become relatively weak positive (Ito *et al*, 2002; Xiao *et al*, 2003). As pointed out by Jiang *et al* (2001), Em18 is not species-specific, but genus-specific. Therefore, the difference between AE and CE in response to Em18 is due to different pathology. The *E. granulosus* metacestode grows in double-walled cysts by endogenous budding, whereas the *E. multilocularis* metacestode grows by exogenous budding. The challenge to produce a synthetic peptide of Em18 is ongoing.

### Cystic echinococcosis (CE)

Serology for detection of CE using Antigen B or Antigen 5 is well-known (Lightowers and Gottstein, 1995). There are some other candidate antigens like cyclophilin (Lightowers *et al*, 1989) and thioredoxin peroxidase (Li *et al*, 2004) and some other component (Li *et al*, 2003). None of these candidate antigens is expected to be species-specific. As mentioned above for AE, all candidate antigens so far reported are genus-specific, but antibody responses differ between AE and CE patients. Among these antigens, summarized by Lightowers and Gottstein (1995), Antigen B is particularly expected to be more useful for detecting CE compared with AE (Mamuti *et al*, 2004). So far as we know, Antigen B is most widely used for serodiagnosis of CE and studied from the viewpoint of biochemistry and molecular biology (Gonzalez *et al*, 1996). The most recent work by Mamuti *et al* (2004) is interesting, since over 80% of confirmed CE cases show strong antibody responses to Antigen B, either native or recombinant produced by *E. granulosus* (recEgAgB8/1) or *E. multilocularis* (recEmAgB8/1), whereas approximately 40% of AE only become positive, although all AE sera examined for this study were positive to Em18. Therefore, we are interested in comparative analysis of pathological, biochemical, and molecular biological aspects of these two antigens in both *E. multilocularis* and *E. granulosus*. Another merit of using recombinant Antigen B is no cross-reaction with cysticercosis, although when native Antigen B or cyst fluid of hydatid cysts of *E. granulosus* are used, it often cross-reacts with cysticercosis patients' sera (Mamuti *et al*, 2002). Antigen B is one of the hydrophobic ligand binding proteins (HLBPs) of cestodes. Due to the poor homology between Antigen B8/1 and *Taenia solium* Antigen B (TsolAg1V1, by Sako *et al*, 2000; Hancock *et al*, 2003), there may be a good explanation for no cross-reaction between echinococcosis and cysticercosis (Mamuti *et al*, 2004).

### PRESENT SITUATION OF AE IN JAPAN

By contrast, routine screening of AE in Hokkaido,

Japan, is based on serology (ELISA) using crude antigens and confirmative serology (immunoblot) using crude antigens to detect massive banding patterns and/or so-called C-protein, as briefly summarized in the seminar in 2000 (Ito *et al*, 2001). This serology, using crude antigens, appears to be not so specific but is expected to be useful for screening (Ito and Craig, 2003). However, the false-positive rate of nonAE cases appears to be too high. According to Sato *et al* (2003), who are the experts in the surgical treatment of AE in Japan, approximately 1% of ongoing crude Antigen-ELISA-positive people might be real AE. This strategy might be re-evaluated, since it does give us little reliable information about exactly how many AE patients exist in Hokkaido, other than AE cases confirmed after surgery. Most AE cases reported by clinicians are not confirmed by surgery. Clinicians can declare and report AE cases by image data, or by serology using crude antigens, or both. So, there might be no direct evidence of AE with pathological confirmation reported by clinicians. It is evident that image analysis requires experience and are not rare misdiagnosed cases of hepatic cysts or hemangioma in Japan (Ito, 2001; Ito *et al*, 2001). Therefore, it is urgent to establish some consultation or evaluation system for clinicians to obtain more reliable information for the diagnosis of AE.

We at AMC have started such a consultation system for the clinician who suspects AE, 5 years ago. Until the end of November 2003, we have had no misdiagnosed AE cases through 40 consultations on AE by clinicians. This consultation is not only for AE, but also CE and NCC, and several other parasitic infections, such as toxocarasis and paragonimiasis, if clinicians suspect them, and ask for our consultation. This consultation is not limited only to Japan, but anywhere in the world. We have such specimens from China, Thailand, Nepal, Indonesia, Malaysia, Australia, the Netherlands, Poland, Turkey, England, the USA, and Ecuador.

National echinococcosis surveillance in Japan has been performed since April 1999, when it was stipulated as a Category IV disease under the Infectious Disease Prevention Law. However, there is no evaluation system for cases reported by clinicians, as mentioned above. As the serology misdiagnosed fascioliasis and CE as AE cases from the main island, Honshu, after 1999, it is not easy to use such serological information without additional confirmation. Most recently, Arai *et al* (2003) working at the Japanese National Institute of Infectious Diseases, tried to evaluate the reported echinococcosis cases through national surveillance. However, as they had no

additional evaluation system for the reported cases, they could not differentiate fake (misdiagnosed) and sound data. It is sure that the local government has tried to establish screening systems for AE and sustainable education in Hokkaido over the past 50 years. We expect that the established serological screening system worked reasonably well when there were relatively many AE patients. But after detection of the majority of AE cases who suffered from early stage environmental contamination, it does not work well. Therefore, we are anxious to establish better evaluation of AE cases.

It is clear that the approximately 400 AE cases reported before 1999 were basically through surgical treatment with pathological confirmation and, therefore, it is the minimum number of AE cases confirmed from Japan (Minagawa, 1997; Doi *et al*, 2000; Ito *et al*, 2003a). However, there is no additional sound evidence on more than 400 AE-suspected people in Hokkaido through serology. We Japanese expect that serological screening is the most functional and feasible method, but ongoing serology for screening is not suitable at this stage. At AMC, we have found AE cases using Em18 serology without any misdiagnosis and followed by pathological confirmation of AE after surgery, which were seronegative by ongoing serological screening (Ito, 2001). In France, an immunoblot kit is commercially available (Liance *et al*, 2000). It is mainly based on detection of Em18, Em16 (Ito *et al*, 1993), and Antigen B using crude antigens. Based on our studies, it is reasonably reliable (Ito *et al*, 2002; Xiao *et al*, 2003). In AMC, we have developed recombinant Em18 and recombinant Antigen B and the sensitivity and specificity of serology using these recombinant antigens have been improved (Sako *et al*, 2002; Ito *et al*, 2003a; Xiao *et al*, 2003; Mamuti *et al*, 2004).

So, the strategy for detection of AE in Japan may change following the WHO recommendation (Pawlowski *et al*, 2001; Ito and Craig, 2003; Ito *et al*, 2003b). The first choice is image analysis to detect abnormality in the liver, with subsequent serology using highly-specific antigens as confirmative serodiagnosis. Even if Japanese people believe in serologic screening, as the Japanese myth of screening in general, we must apply better, much more reliable antigens for screening. Based on our results from international collaboration projects as blind tests (reviewed by Ito, 2002a; Ito and Craig, 2003; Ito *et al*, 2003c; McManus *et al*, 2003; Zhang *et al*, 2003), we expect that purified and/or recombinant Em18 and Antigen B are much more reliable diagnostic antigens, sensitive for identifying echinococcosis, and expected

to be useful for screening, as well. We have detected 2 AE cases, which were seronegative by ongoing serology using crude antigens, as antibody-positive to Em18 and confirmed pathologically after surgery (Kitada *et al*, 2001; Ito *et al*, unpublished), and, *vice versa* a non-AE case strongly positive by ongoing confirmative immunoblot serology using crude antigens for several years was thoroughly negative to Em18 and was confirmed as hepatic cyst, in 2003 (Aoki *et al*, in preparation). So far we have conducted studies even as blind tests, and there were no false-positive AE cases (Ito *et al*, 2002; Xiao *et al*, 2003; Mamuti *et al*, 2004).

Although we have no doubt of the great sustained effort by the local government in Hokkaido to establish the screening system for AE, we prefer to follow the WHO recommendation (Pawlowski *et al*, 2001). As many AE cases have been detected through the screening system, it might evolve into a different phase, that of detecting rare AE cases. Even if AE cases might increase in the future due to the high contamination of foxes, as shown by Romig *et al* (1999), and in this symposium (Romig, 2004), image diagnosis is the most feasible in many hospitals in Japan and so, based on some picture of hepatic abnormality, such cases may better be checked using specific antigens to reduce the confusion, anxiety, and expense for persons whose sera give false-positive reactions with crude antigens (Ito *et al*, 2003c).

#### ECHINOCOCCOSIS IN DEFINITIVE ANIMAL HOSTS

##### *Echinococcus multilocularis* and *E. granulosus*

The definitive hosts of *E. multilocularis* are wild canid animals, mainly foxes. Reasonably reliable methods exist to obtain screening information by copro-antigen test (Allan *et al*, 1992; Nonaka *et al*, 1996; Deplazes *et al*, 1999). This technique, to detect copro-antigens of *Echinococcus* spp, is primarily genus-specific or family-specific but not species-specific, and is useful for primary screening, particularly for wild animals, but is insufficient for identifying species. Therefore, it is important to establish species-specific tests, since there are endemic areas for both *E. multilocularis* and *E. granulosus* in the northern hemisphere (Craig *et al*, 1992; Ito *et al*, 2003a) and dogs as companion animals may become more important definitive hosts for *E. multilocularis* to transmit the disease to humans (Craig *et al*, 2000).

Because the risk of human infection by dogs could be higher than that from foxes in China and in other countries (Craig *et al*, 2000), detection of copro-DNA

as well as classical morphological confirmation of echinococcal adult worms, the most reliable direct evidence, is required for species-specific confirmation based on screening of copro-antigen tests. There is nothing better than direct evidence through confirmation of adult worms. On this point, it has been highly appreciated that the local government has been carrying out necropsies of foxes in Hokkaido, every year.

As such necropsy of foxes is very hard work, an alternative screening system to detect copro-antigens has been developed, as mentioned above. To avoid undue public anxiety, especially where companion animals are concerned, it must be stressed that any definitive diagnosis of *E. multilocularis* in dogs and cats be based on certain evidence: (1) the morphological detection of adult worms expelled after purging with arecoline (not after dosing with praziquantel) of animals positive with copro-antigen tests as used to be applied for detection of dogs infected with *E. granulosus*, and/or (2) the detection of species-specific DNA in fecal samples, or from eggs or adult worms present in the feces (Ito, 2002b; Ito and Craig, 2003). Sustainable public health education is only available through evidence-based medicine and science.

This is the real need for establishing a surveillance system with evaluation of case reports and for the future establishment of a better quality of life for residents living in Hokkaido.

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